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TITLE: Preclinical and Clinical Investigation of the Impact of Obesity on Ovarian Cancer Pathogenesis

PRINCIPAL INVESTIGATOR: **Victoria Bae-Jump, MD, PhD**

CONTRACTING ORGANIZATION:
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14. ABSTRACT

The metabolic consequences of obesity may be critical in the development of ovarian cancer (OC), resulting in biologically different cancers than those that arise in leaner women. This may occur through aberrant modulation of mTOR signaling, given that alterations in this pathway are common in both obesity and OC. Thus, obese OC patients may derive increased benefit from chemotherapeutic agents related to inhibition of this pathway, such as mTOR inhibitors (everolimus) or metforrnin. We demonstrate that the obese state can promote tumor progression in the KpB mouse model of OC. The ovarian tumors that arose in the obese mice were genomically and metabolically different from those that arose in non-obese mice. Alterations in gene expression were found with elevated BMI in human serous OC tumors in The Cancer Genome Atlas (TCGA) database. Many of these genes were related to lipid metabolism, specifically apolipoproteins. Metformin but not everolimus was more efficacious in the inhibition of tumor growth in obese *versus* non-obese mice. For our *in vitro* studies, metformin and everolimus had similar effects on proliferation, inhibition of mTOR signaling and glycolysis but opposite effects on glucose uptake, which may contribute to metforrnin's enhanced anti-tumorigenic effects in the setting of obesity.

15. SUBJECT TERMS
Ovarian cancer, mTOR pathway, mTOR inhibitors, metforrnin, obesity, genomics, metabolomics

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September 15, 2014

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US Army Medical Research Acquisition Activity
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301-619-2090
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Dear Ms. Dellinger,

We are requesting a no cost extension for Dr. Vickie Bae-Jump's proposal entitled, "Pre-clinical and Clinical Investigation of the Impact of Obesity on Ovarian Cancer Pathogenesis" proposal number W81 XWH-12-1-0426, for a one year period with new end date of 09/24/2015. This request follows the receipt of an annual progress report (attached) by Dr. Bae-Jump. Funds remaining will follow the intended purposes of the original budget. Please refer to the conclusion section for our work to be conducted during the extension period.

We appreciate your support. If you have any questions or need additional information, please contact me at 919-966-2629, or my email at mark_kramer@med.unc.edu.

Sincerely,

Mark Kramer
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for

Barbara Entwisle
Vice Chancellor for Research

(1) INTRODUCTION

Obesity leads to elevated incidence and worse outcomes for ovarian cancer (OC) (1-18). We postulate that the metabolic consequences of obesity may be crucial in the development of OC, resulting in biologically different cancers than those that arise in normal weight women. This may occur through aberrant modulation of mTOR signaling, given that alterations in this pathway are common in both disease processes. Thus, obese OC patients may derive increased benefit from chemotherapeutic agents related to inhibition of this pathway, such as mTOR inhibitors or metformin. This proposal will address this question by investigating the impact of obesity on the proliferative and metabolic effects of mTOR inhibitor and metformin treatment in three model systems: *in vitro* using OC cell lines; *in vivo* using a novel serous ovarian tumor murine model; and in a pilot clinical trial. The role of obesity in OC initiation and promotion will be evaluated through comprehensive cross-species genomic and metabolomic analysis with the goal of identifying common genetic or metabolic biomarkers associated with obesity-driven cancers and differential response to treatment in the obese and non-obese state. If our hypothesis is true, optimization of OC treatment may need to encompass tumor characteristics as well as obesity status.

(2) KEYWORDS: Ovarian cancer, mTOR pathway, mTOR inhibitors, metformin, obesity, genomics, metabolomics

(3) OVERALL PROJECT SUMMARY

Task 1 (Aim 1): To assess the effect of the mammalian target of rapamycin (mTOR) inhibitor everolimus and metformin on key metabolic pathways in human ovarian cancer cell lines under high and low glucose conditions.

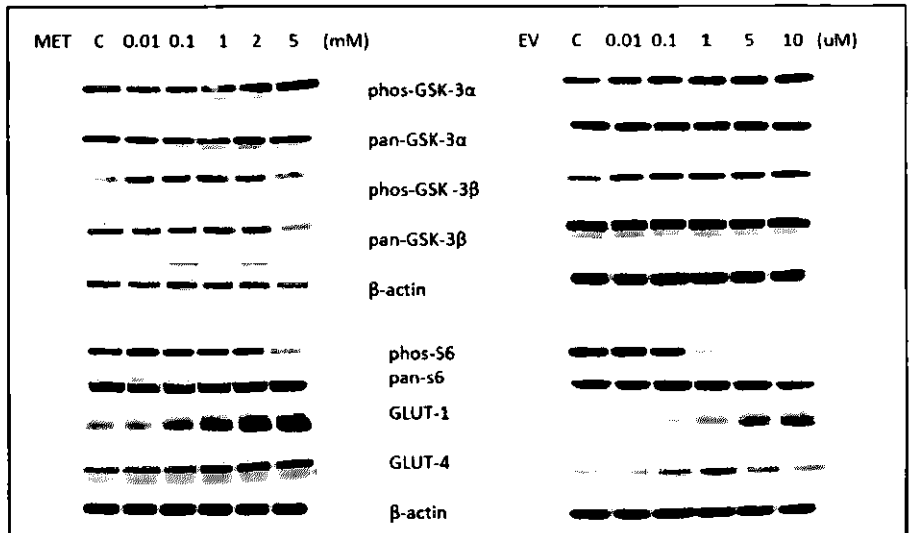


Figure 1. Metformin and everolimus increased phosphorylation of GSK-3β and GSK-3α and reduced phosphorylation of S6 expression in a dose dependent manner in the SKOV3 cell line after 24 hours of treatment, as assessed by Western immunoblotting analysis. In parallel, metformin and everolimus increased GLUT1 and GLUT4 expression. Similar effects were seen in the IGROV1 and OV5 cell lines.

Overweight and obese states may be linked to OC through nutrient-sensitive signaling cascades, such as the insulin/insulin growth factor (IGF) and PI3K/Akt/mTOR pathways (19-23). Hyperinsulinemia, insulin growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) levels are important in OC development and progression in experimental and epidemiological studies (24-27). Signaling through IGF-1R leads to activation of the PI3K/Akt/mTOR pathway, and components of this pathway are often mutated, amplified or aberrantly expressed in OCs (28-33). Thus, mTOR inhibitors, such as everolimus (also known as RAD001), as a targeted therapy for OC are currently being actively investigated in Phase 1, 2 and 3 clinical trials (34-36).

Metformin is an anti-diabetic medication from the biguanide class that is widely used as the first line treatment of type 2 diabetes. Mounting epidemiological evidence suggests that metformin use lowers cancer risk and reduces cancer deaths among diabetic patients (37-39), including OC (40-42). Metformin is believed to have both indirect and direct effects on tumor growth, and it is unknown which of these effects are most important for metformin's anti-tumorigenic benefits. Its indirect effects are likely to be due to inhibition of hepatic gluconeogenesis, resulting in an improvement in insulin sensitivity and a reduction in blood glucose and circulating insulin levels, which may lead to decreased growth factor-stimulated tumor growth. On a direct level, metformin may affect tumor growth by activation of adenosine monophosphate-activated protein kinase (AMPK), its intracellular target for anti-diabetic effects, which leads to the regulation of multiple downstream

signaling pathways that control cellular proliferation, including inhibition of the mammalian target of rapamycin (mTOR) pathway.

Given that we have previously shown that metformin and mTOR inhibitors are potent inhibitors of OC cell proliferation (43, 44), we wanted to assess whether these agents also had an effect on glucose metabolism in OC cell lines. Western immunoblotting analysis revealed that treatment with metformin and the mTOR inhibitor, everolimus, increased facilitative glucose transporter 1 and 4 (GLUT-1 and GLUT-4) expression and increased phosphorylation of glycogen synthase kinase-3 alpha and beta (GSK-α and GSK-β) (**Figure 1**). In parallel, metformin and everolimus inhibited the mTOR pathway, as evidenced by decreased phosphorylation of its downstream target, S6 (**Figure 1**). These results suggest that treatment with metformin and everolimus potentially drives glucose metabolism and uptake in OC cells, despite blunting proliferation.

Subsequently, the effects of metformin and everolimus treatment on cell proliferation and apoptosis was assessed under normal, low and high glucose conditions in OC cell lines. Our goal was to mimic the obese-diabetic state *in vitro* through the use of high physiologic glucose. As expected, metformin and everolimus inhibited proliferation in both cell lines under normal glucose conditions (**Figure 2A and 2B**), through G1 cell cycle

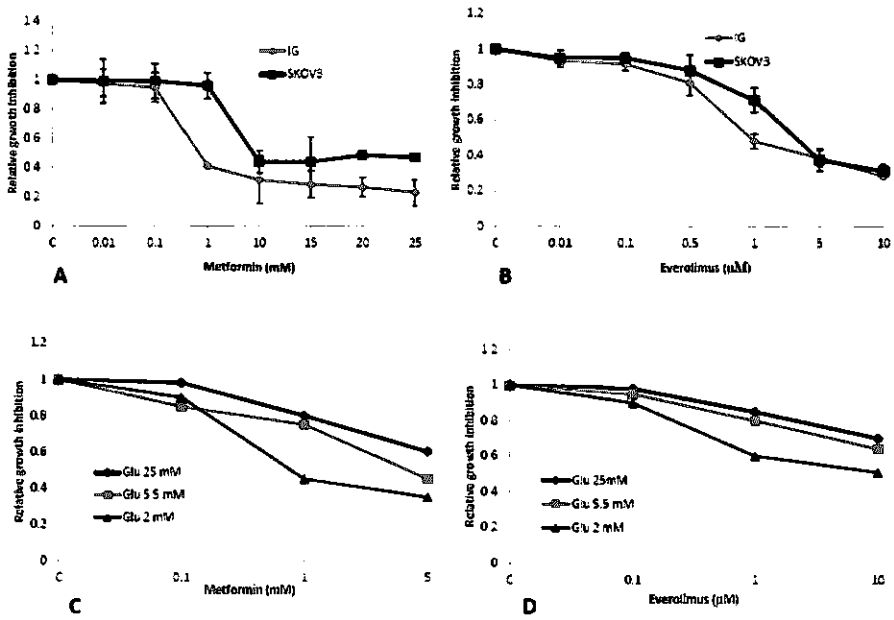


Figure 2. The SKOV3 and IGROV1 (IG) cell lines were treated with metformin and everolimus under low glucose (2 mM), normal glucose (5.5 mM) or high glucose (25 mM) conditions for 72 hours. Cell growth was determined by MTT assay. Metformin and everolimus inhibited proliferation in both cell lines under normal glucose conditions (**A and B**). Metformin and everolimus were found to be more effective in the inhibition of proliferation under low versus high glucose concentrations (**C and D**). Similar results were also found for the OV5 cell line.

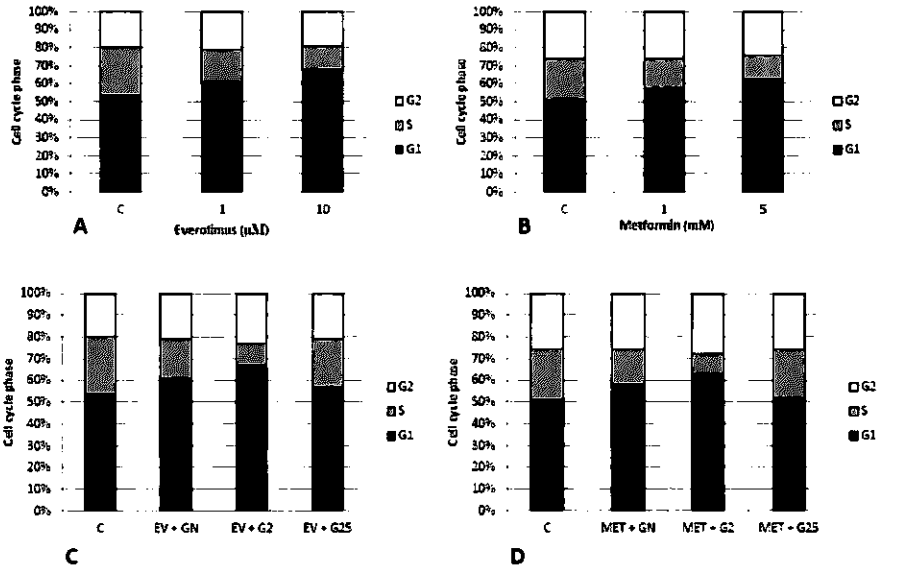


Figure 3. Metformin and everolimus induced G1 cell cycle arrest in the SKOV3 cell line (**A and B**). The SKOV3 cell line was treated with metformin (1 mM) or everolimus (1 uM) under low glucose (2 mM, G2), normal glucose (5.5 mM, GN) or high glucose (25 mM, G25) conditions for 24 hours (**C and D**). Metformin and everolimus were found to be more effective in G1 cell cycle arrest under low versus high glucose conditions. Cell cycle phase analysis was assayed by cellometer. Similar results were also found with the IGROV1 and OV5 cell lines. *EV=everolimus, MET=metformin

arrest (**Figure 3A and 3B**). Metformin and everolimus were found to be more effective in the inhibition of proliferation under low versus high glucose concentrations (**Figure 2C and 2D**), as also evidenced by enhanced G1 cell cycle arrest (**Figure 3C and 3D**). In addition, metformin and everolimus were found to be more effective in the induction of apoptosis under low versus high glucose concentrations (**Figure 4**). For all of these experiments, the effects of metformin and everolimus under normal glucose conditions was intermediary between low and high glucose levels. These results were opposite to what we had predicted; we had hypothesized that metformin and everolimus would be more effective under high glucose as opposed to low and normal glucose conditions.

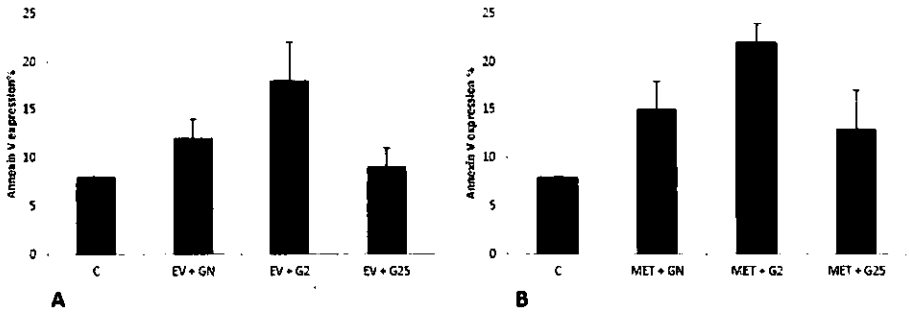


Figure 4. The SKOV3 cell line was treated with metformin (1 mM; B) or everolimus (1uM; A) under low glucose (2 mM; G2), normal (5.5 mM; GN) or high glucose conditions (25 mM; G25) for 24 hours. Metformin and everolimus were found to be more effective in the induction of apoptosis under low versus high glucose concentrations. Annexin V expression, a marker of apoptosis, was assessed by cellometer. Similar results were also found for the IGROV1 and OV5 cell lines. *EV=everolimus. MET=Metformin

Given these unexpected findings, we did examine the effects of differing glucose conditions (i.e. low, normal and high) alone on AMPK activation and mTOR pathway inhibition in the OC cells. As the concentration of glucose increased, we found that phosphorylation of AMPK decreased and phosphorylation of S6 increased, suggesting increased proliferative capacity and hyperactivation of the mTOR pathway with high physiologic glucose (**Figure 5**). Metformin and everolimus were effective under all glucose conditions in the OC cells; however, we observed heightened sensitivity under low versus high glucose conditions. Thus, we postulate that high glucose levels may override some of the anti-proliferative effects of both of these agents *in vitro*, and that cells exposed to low glucose may have blunted proliferative capacity and may be more inherently susceptible to metformin and everolimus.

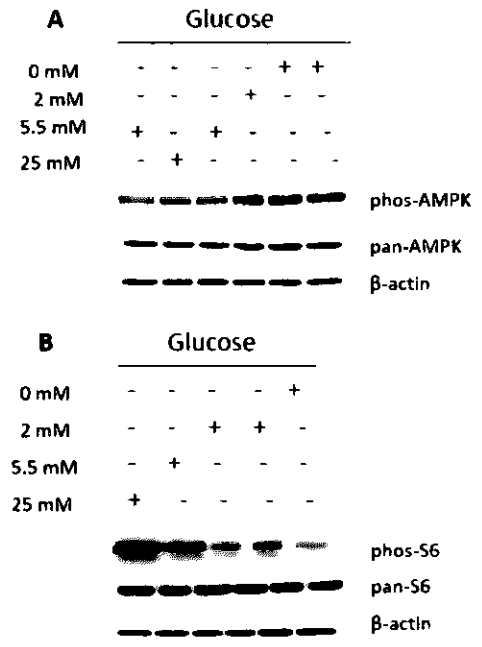


Figure 5. The SKOV3 cell line was exposed to different glucose concentrations for 24 hours. Phosphorylated (phos)-AMPK and S6 and pan-AMPK and S6 expression was determined by Western immunoblotting analysis. As the concentration of glucose increased, phosphorylation of AMPK decreased and phosphorylation of S6 increased. Similar results were found for the IGROV1 and OV5 cell lines.

The effect of metformin versus everolimus on glycolysis and glucose uptake was assessed in the OC cell lines (**Figure 6**). Glucose increased ATP production in the OC cells which was then reversed by treatment with either metformin or everolimus. As expected, metformin increased glucose uptake and everolimus decreased glucose uptake in the OC cell lines. Thus, metformin and everolimus both decrease proliferation and glycolysis in OC cells but have opposite effects on glucose uptake.

In the upcoming report period, Western immunoblotting will be performed to assess the expression of the downstream signaling targets of everolimus and metformin in OC cell lines under high, normal and low physiologic glucose concentrations. This work should provide a detailed examination of glucose metabolism along with proliferation to determine the interrelationship of everolimus and metformin treatment on cell metabolism and growth under varying metabolic environments such as high and low glucose conditions.

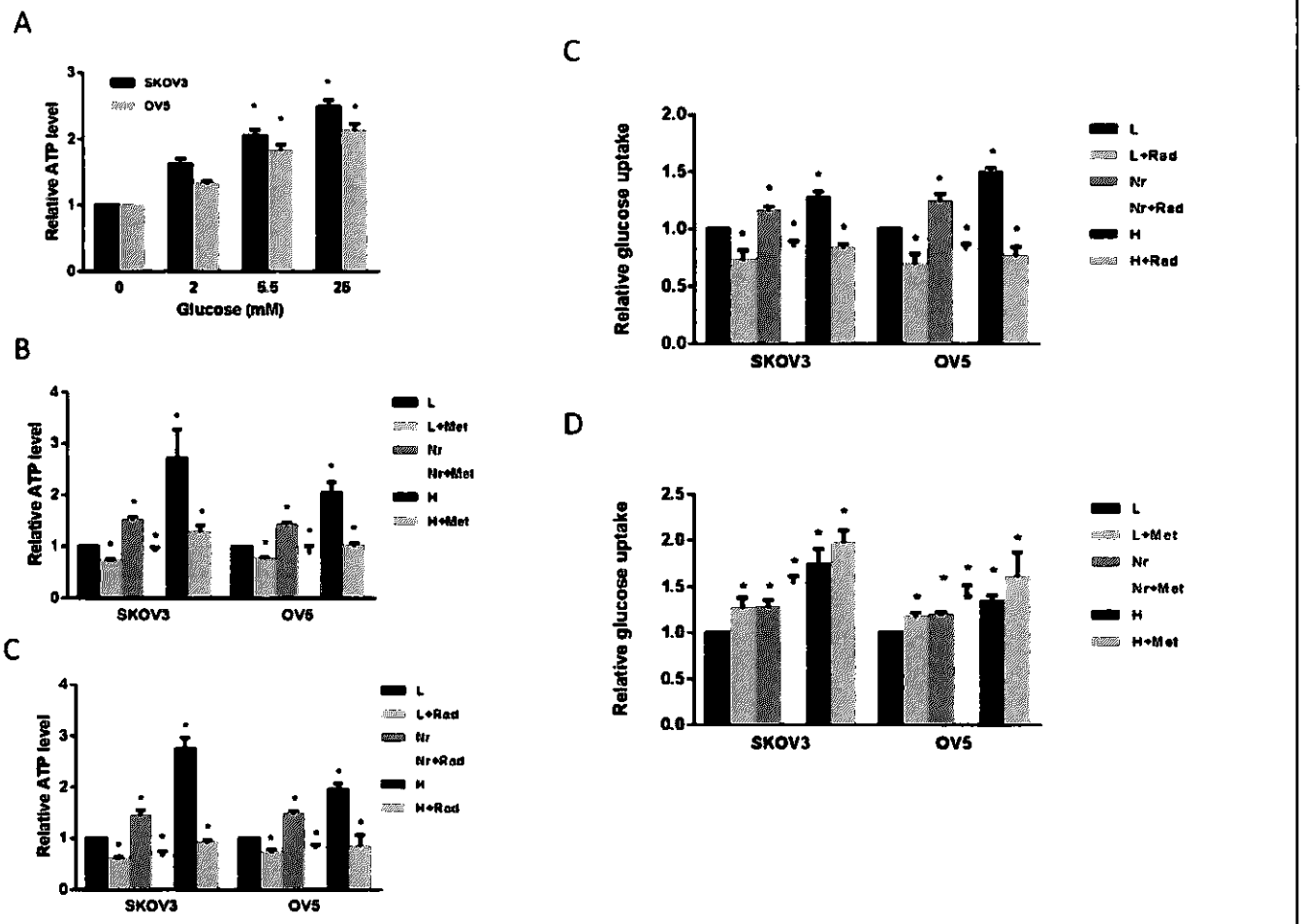


Figure 6. The effect of metformin and everolimus (RAD001) on glycolysis and glucose uptake in ovarian cancer cell lines. The SKOV3 and OV5 cell lines were treated with either metformin or everolimus for 18 hours. Glucose increased ATP production in OV5 and SKOV3 cells (**A**). Metformin and everolimus decreased ATP production induced by glucose (**B and C**). Metformin increased glucose uptake and everolimus decreased glucose uptake in OV5 and SKOV3 cells after 24 hours treatment (**D and E**). *Rad=RAD001 or everolimus, Met=Metformin

Task 2 (Aim 2): To compare tumor latency, growth and response to treatment with the mTOR inhibitor everolimus and metformin in lean and obese K18-gT$_{121}^{+/-}$;p53$^{fl/fl}$;Brca1$^{fl/fl}$ (KpB) mice using genomics and metabolomics to identify pathways and biomarkers associated with therapeutic success.

We have previously described a unique serous OC mouse model that specifically and somatically deletes the tumor suppressor genes, Brca1 and p53, and inactivates the retinoblastoma (Rb) proteins in adult ovarian surface epithelial cells (KpB

Table 1. Diet-induced metabolic characteristics in non-obese and obese KpB mice.

	Non-Obese	Obese	p-value
Weight (gms)	31.14 ± 5.26	50.71 ± 16.73	p=0.0003
Glucose (mg/dl)	186.81 ± 26.99	214.38 ± 58.11	p=0.053
% Fat	3.28 ± 1.51	19.58 ± 7.88	p=0.00001
% Lean	22.89 ± 2.11	28.66 ± 5.24	p=0.0006
N=14 mice per group. Mean ± SD. % Fat or % lean = each mass/total body mass as measured by MRI.			

mouse model) (45, 46). Subsequently, KpB mice were subjected to a 60% calories-derived from fat in a high fat diet (HFD) versus 10% calories from fat in a low fat diet (LFD) to induce diet-induced obesity (N=14/group) starting at 6 weeks of age and until sacrifice. After 8 months of exposure to the HFD or LFD, obese mice weighed significantly greater than non-obese mice (p=0.003, Table 1). There was no effect of HFD on non-fasted blood glucose levels or diabetes onset in KpB mice over the course of the diet (**Table 1**). Body composition was significantly altered in obese KpB mice compared to non-obese controls. Percent body fat was six-fold greater in obese mice (Table 1, p=0.0001), while percent lean mass increased by 25% (p=0.0006, Table 1). The ovarian tumors were tripled in size in the obese mice as compared to non-obese mice (mean size of 3.7 cm^2 versus 1.2 cm^2, **Figure 7**, p=0.0065). This suggests that obesity can promote tumor progression in this KpB mouse model of OC.

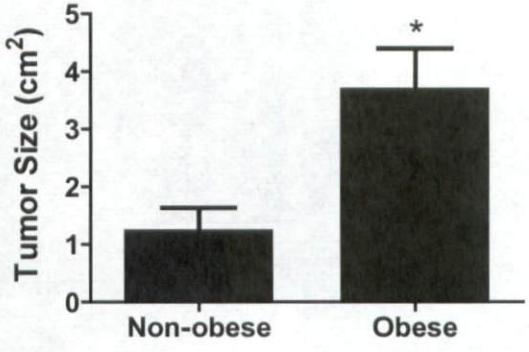


Figure 7. Obesity increases tumor size in KpB mice. KpB mice were fed low fat or high fat diets to induce obesity for 6 months during tumorigenesis. Comparison of tumor size from non-obese and obese mice (N=14). *p=0.0065.

Obesity was found to induce genomic differences between the obese and non-obese ovarian tumors. 439 genes were found to be significantly up-regulated (417 genes) or down-regulated (22 genes) in the ovarian tumors from obese KpB mice versus non-obese mice (FDR<0.2). **Figure 8** is a heat map of 131 genes up- and down-regulated at a FDR<0.1. Many metabolically relevant genes were significantly upregulated in the ovarian tumors from the obese versus non-obese mice, such as lipocalin (2.7 fold), fatty acid amide hydrolase (2.7 fold), fatty acid 2-hydroxylase (2.2 fold), glycerol-3-phosphate acyltransferase (1.5 fold), protein phosphatase (1.2 fold), AMP deaminase 3 (1.6 fold), and protein kinase C (1.7 fold). Arginase 1 was the most upregulated gene (7.3 fold) and plays a role in the urea cycle, tissue remodeling and inflammation. Other upregulated genes identified in the ovarian tumors from the obese mice were related to cell adhesion, including neurotrimin (2.2 fold) and desmoglein 1-alpha (2.0 fold). Increased expression of histone 1 (2.3 fold) and endothelin-1 (5.8 fold) were also associated with obesity in the KpB mouse model. Another gene upregulated 3 fold was ectonucleoside triphosphate diphosphohydrolase. Heparan sulfate (glucosamine) 3-O-sulfotransferase 1 was upregulated 6 fold and regulates heparan sulfate production which is involved in developmental processes, angiogenesis, blood coagulation and tumor metastasis. The serotonin transporter solute carrier family 6 member 4 (Slc6a4) was upregulated 5.4 fold by obesity. Important downregulated genes included spermidine synthase, an enzyme in spermidine synthesis and thrombospondin 4, an extracellular glycoprotein known to have roles in cellular migration, adhesion, attachment and proliferation. In the ovarian tumors from the obese versus non-obese mice, DAVID functional annotation analysis revealed significant enrichment in "phospoholipid binding" (EASE score of 0.008), "regulation of apoptosis" (EASE score of 0.014), "lipid binding" (EASE score of 0.015), "endopeptidase activity" (EASE score of 0.03) and "cell-cell signaling" (EASE score of 0.44) for those identified genes.

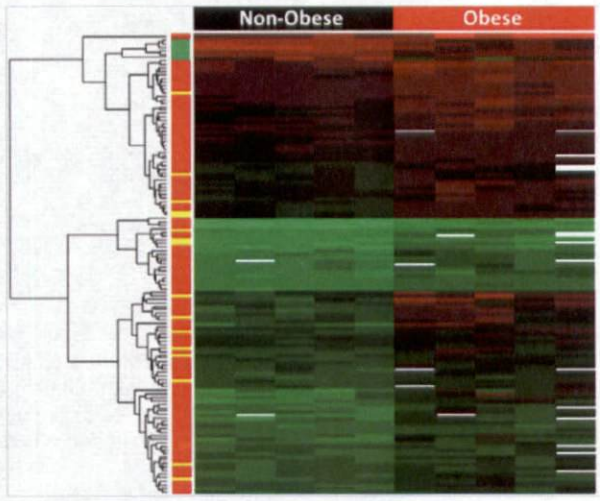


Figure 8. Genomic differences between ovarian tumors from obese versus non-obese KpB mice reveal alterations in metabolically relevant genes. Heat map representation of 131 genes significantly up- or down-regulated in ovarian tumors from obese versus non-obese KpB mice (FDR<0.1). Many metabolically relevant genes, such as lipocalin, fatty acid amide hydrolase, ectonucleoside triphosphate diphosphohydrolase, fatty acid 2-hydroxylase, glycerol-3-phosphate acyltransferase, protein phosphatase, protein kinase C and AMP deaminase 3, were upregulated in obese tumors.

Table 2. Metabolic alterations in tumors from non-obese and obese KpB mice.

Compound name	VIP [a]	p [b]	Fold Change (non-obese/obese) [c]	Analysis method	Identification Method [e]
N-Glycylproline	2.27	0.0043	1.95	LC-ES+	Std
Oxidized glutathione	2.25	0.0047	3.45	LC-ES+	Std
N-Acetylaspartic acid	2.22	0.0059	2.31	LC-ES-	HMDB
Vanillactic acid	2.17	0.0079	2.23	LC-ES+	HMDB
3-amino-2-piperidone	2.14	0.0099	1.75	GCTOF	NIST
Cytidine	2.10	0.0122	4.52	LC-ES+	Std
Cytosine	2.05	0.0158	4.11	LC-ES+	Std
LysoPC(16:1(9Z))	1.99	0.0205	1.83	LC-ES+	HMDB
8-Hydroxy-deoxyguanosine	1.97	0.0230	2.45	LC-ES+	HMDB
Adenosine monophosphate	1.94	0.0257	1.61	LC-ES-	HMDB
Arginine	1.93	0.0268	1.93	LC-ES+	Std
Gluconolactone	1.89	0.0311	2.97	LC-ES+	Std
Glutathione	1.89	0.0313	3.10	LC-ES+	Std
Glutamate	1.89	0.0318	1.52	GCTOF	Std
Guanosine diphosphate	1.82	0.0404	2.39	LC-ES-	HMDB
Cytidine	1.81	0.0424	4.97	GCTOF	NIST
Inodxyl glucuronide	1.80	0.0439	3.05	LC-ES+	HMDB
Phenylethanolamine	1.80	0.0446	1.69	GCTOF	NIST
Succinic acid	1.78	0.0465	1.90	GCTOF	Std
5-Hydroxyindoleacetic acid	1.76	0.0498	1.85	LC-ES+	HMDB

[a] variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0; [b] p value was calculated from Student's t Test; [c] Fold change with a value larger than 1 indicates a relatively higher concentration in tumors from non-obese (low fat diet-fed) KpB mice, while a value less than 1 means a relatively lower concentration as compared to tumors from obese (high fat diet-fed) KpB mice. [e] The metabolites were identified by in-house library (Std), NIST library (NIST) or HMBD database (HMDB).

Metabolic differences were also found between the ovarian tumors from obese and non-obese KpB mice. Principle component analysis defined a clear separation between obese and non-obese. Differentiating metabolites were selected with the criteria of the variable importance in the projection (VIP) value>1 and p value (Student's t test) lower than 0.05. Twenty metabolites were identified using this criteria, all of which were upregulated in the ovarian tumors of the non-obese versus obese KpB mice (**Table 2**).

Metabolites involved in inflammatory signaling and protein/collagen metabolism were down-regulated in the ovarian tumors of obese mice as compared to non-obese mice, including arginine (p=0.0268), N-glycylproline (p=0.0043) and 3-amino-2-piperidone (p=0.0099). Components and markers of oxidative stress were also downregulated in the tumors from obese mice: glutathione (p=0.0313), oxidized glutathione (p=0.0047), gluconolactone (p=0.0311) and 8-hydroxy-deoxyguanosine (p=0.0230). Lower levels of nucelotides (i.e. cytidine (p=0.0122 and p=0.0424), cytosine (p=0.0158), guanosine diphosphate (GDP, p=0.0404)) and adenosine monophosphate (AMP, p=0.0257) were detected with obesity. The serotonin metabolite, 5-hydroxyindoleacetic acid (5HIAA, p=0.0498), and the catecholamine metabolites, vanillactic acid (p=0.0079) and phenylethanolamine (p=0.0446), were found to be lower in the ovarian tumors of obese versus non-obese mice. Glutamate (p=.0318), N-acetylaspartic acid (p=0.0059) and succinic acid (p=0.0465) are involved in energy metabolism, and were decreased in the ovarian tumors of obese KpB mice as compared to their non-obese counterparts. LysoPC(16:1(9Z)) (p=0.0205), a lysophospholipid, and the metabolite of a toxic intermediate, inodxyl glucuronide (p=0.0439), were also lower in the ovarian tumors from obese animals.

Slides have been made from these tumors and are undergoing immunohistochemical analysis to assess for differences in signaling targets of the PI3K/Akt/mTOR pathway. To date, we have not found significant differences between phosphorylation of mTOR or AMPK or the facilitative glucose transporter-1 (GLUT1), but we are also assessing other downstream targets of the metformin/mTOR pathway, including Akt, S6, 4EBP-1 as well as other GLUTs. These findings will be important for the cross-species comparisons between obese and non-obese mice and women in Task 3 (Aim 3).

Metformin (200 mg/kg/day, orally) inhibited tumor growth in the KpB mice fed a LFD and a HFD (n=8-10 animals per group), after one month of treatment (**Figure 9**). In the mice fed a HFD, metformin decreased tumor growth by 60% compared to control animals. Tumor growth was only decreased by 32% in the mice fed a LFD. A comparison of the anti-tumorigenic effects of metformin in mice fed a LFD versus a HFD demonstrated that metformin was more efficacious in mice on the HFD (p=0.003), suggesting that metformin may be more beneficial in the setting of obesity. Immunohistochemical analysis was performed of the ovarian tumors after treatment with metformin or control to assess for effects on proliferation, apoptosis and downstream targets of the mTOR pathway (**Figure 10**). As compared to control-treated mice, metformin decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors for obese (HFD) and non-obese (LFD) KpB mice. In addition, metformin increased phosphorylation of AMPK and decreased phosphorylation of S6, a downstream target of the mTOR pathway.

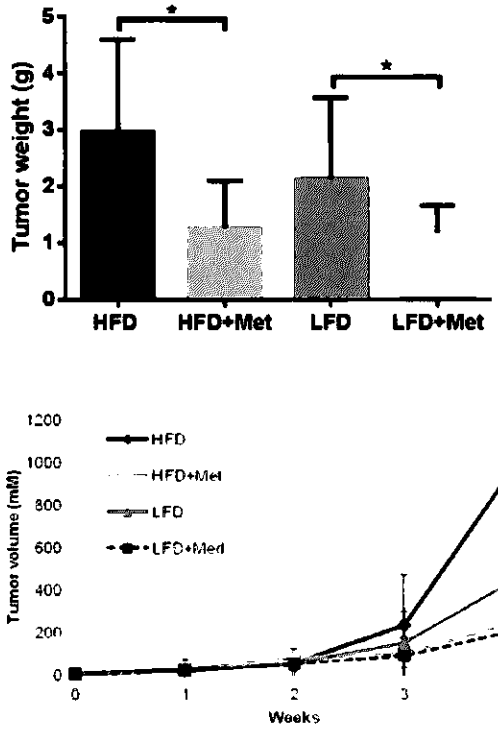


Figure 9. Metformin inhibited ovarian tumor weight and volume in obese and non-obese KpB mice. In obese mice, metformin decreased tumor weight/volume by 60% compared to obese controls. Tumor weight/volume only decreased by 32% in non-obese mice compared to non-obese controls. A comparison of the anti-tumorigenic effects of metformin on ovarian tumors from non-obese and obese mice demonstrated that metformin was more efficacious in obese mice (p=0.003), suggesting that metformin may be more beneficial in the setting of obesity. *Met=Metformin

Everolimus (3 mg/kg/day, intraperitoneally) inhibited tumor growth in the KpB mice fed a LFD and a HFD (n=11-13 animals per group), after one month of treatment (**Figure 11**). In the mice fed a HFD, everolimus decreased tumor weight and volume by 39% compared to control animals. Tumor weight and volume was decreased by 38% in the mice fed a LFD. Thus, in contrast to metformin, everolimus had equivalent effects on the inhibition of ovarian tumor growth in the obese (HFD) and non-obese (LFD) KpB mice. Immunohistochemical analysis was performed of the ovarian tumors after treatment with everolimus or control to assess for effects on proliferation, apoptosis and downstream targets of the mTOR pathway (**Figure 12**). As compared to control-treated mice, everolimus decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors for obese (HFD) and non-obese (LFD) KpB mice. In addition, everolimus decreased phosphorylation of S6, a downstream target of the mTOR pathway.

Tumor has been collected post-metformin/control and post-everolimus/control treatment in the HFD and LFD groups and has been submitted to Metabolon for metabolomic analysis. Preliminary findings indicate that response to everolimus appeared to be essentially similar regardless of whether the ovarian tumor was from a an obese versus non-obese KpB mouse. In contrast, metformin seemed to show more differences between obese and non-obese mice and seemed to have a greater tendency to have a fold-change that was opposite between the obese and non-obese tumor groups. As examples, this trend is shown with succinate and fumarate of the mitochondrial tricarboxylic acid (TCA) cycle and with biopterin metabolites.

Metformin is known to block mitochondrial complex I. The depletion of succinate and build-up of fumarate are consistent with the inhibition of complex I and suggests that complex II continued to operate in the presence of metformin; however, these effects on the TCA cycle were only seen in the obese mice treated

with metformin (**Table 3**). The biopterins are precursors for the redox co-factor tetrahydrobiopterin (tBH4) which is needed for phenylalanine hydroxylase. Oxidized derivatives of the essential redox co-factor tBH4, biopterin and dihydrobiopterin, were elevated in metformin-treated tumors from obese mice but decreased in tumors from non-obese mice (**Table 4**). Since BH4 is a required co-factor for enzymes such as the pro-angiogenic endothelial nitric oxide synthase (eNOS), we hypothesize whether the shift toward oxidized pterin species indicates an intra-tumor environment less suitable for NO production. These differential effects of metformin on the ovarian tumors from obese and non-obese mice were not seen for everolimus (**Table 3 and 4**). We are awaiting the final report from Metabolon and will include the complete metabolomic data analysis in our next progress report.

RNA has been extracted from these tumors post-metformin treatment and will be submitted for genomic analysis. Serum has been collected from these mice pre- and post-treatment for (1) measurement of glucose, leptin, insulin and adiponectin, and (2) metabolomic analysis.

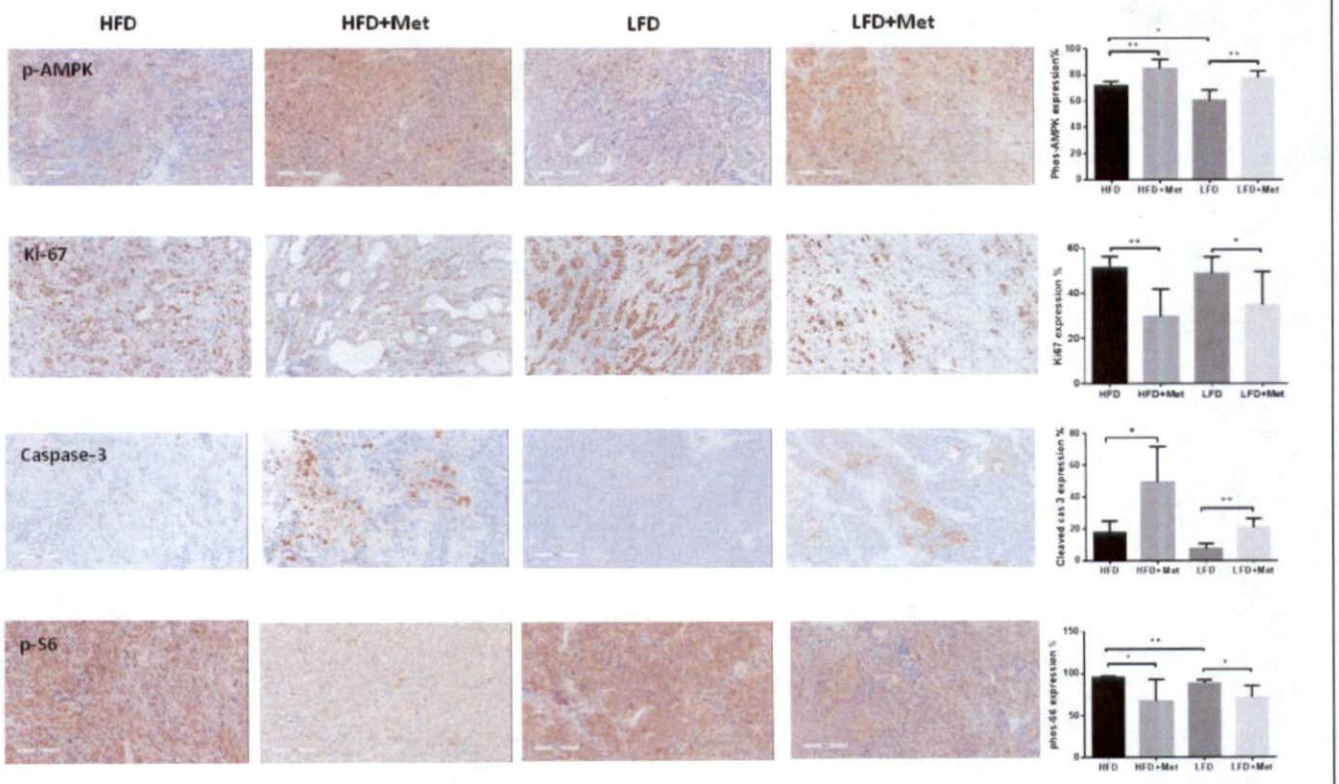
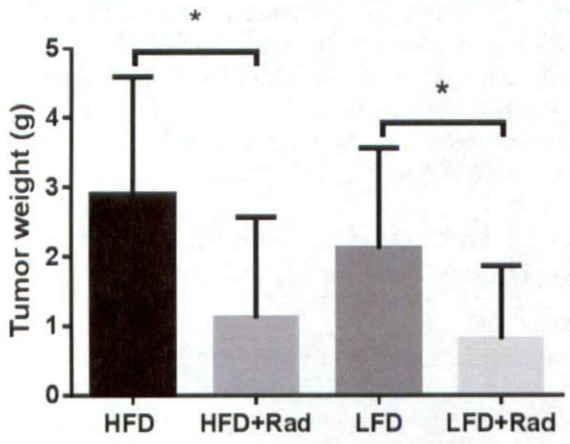


Figure 10. The effects of metformin on proliferation, apoptosis, AMPIK and the mTOR pathway. Metformin decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors from obese (HFD) and non-obese (LFD) KpB mice. In addition, metformin increased phosphorylation of AMPK and decreased phosphorylation of S6, a downstream target of the mTOR pathway. These effects were more pronounced in the ovarian tumors from the obese (HFD) mice than the non-obese (LFD) mice. *Met= Metformin



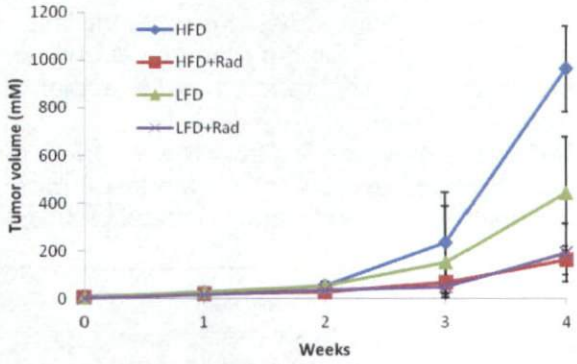


Figure 11. Everolimus (RAD001) inhibited ovarian tumor weight and volume in obese and non-obese KpB mice. In obese mice, metformin decreased tumor weight/volume by 39% compared to obese controls. Tumor weight/volume only decreased by 38% in non-obese mice compared to non-obese controls. *Rad= RAD001

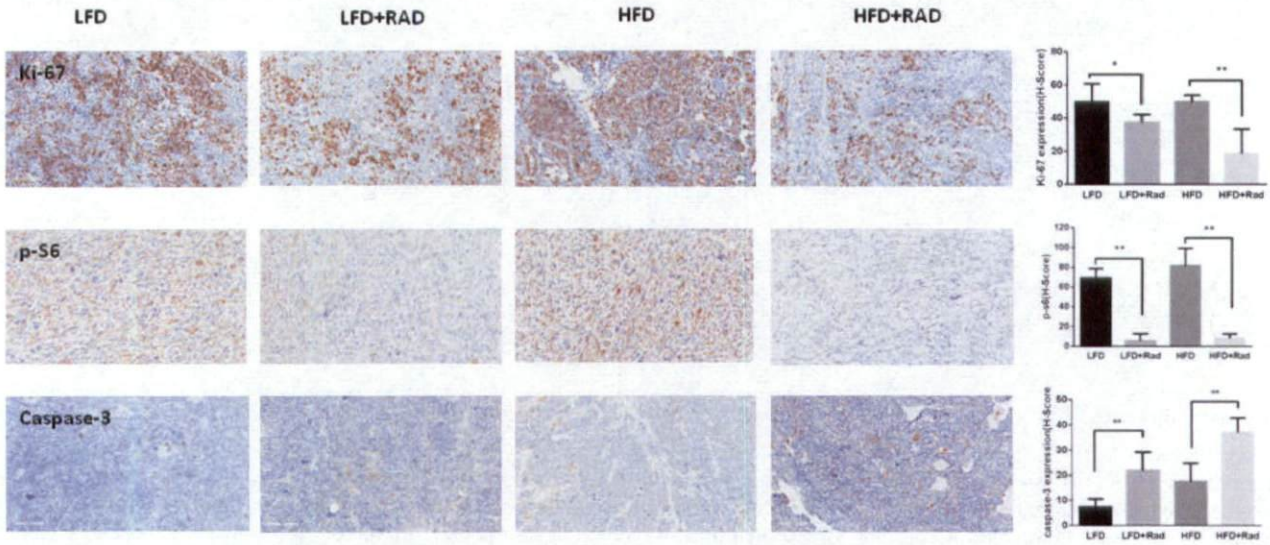


Figure 12. The effects of everolimus (RAD001) on proliferation, apoptosis, AMPIK and the mTOR pathway. Everolimus decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors for obese (HFD) and non-obese (LFD) KpB mice. In addition, everolimus decreased phosphorylation of S6, a downstream target of the mTOR pathway. *RAD=RAD001

Table 3. The Effects of Metformin and Everolimus on the TCA Cycle in the Ovarian Tumors from Obese and Non-Obese Mice * Met= Metformin, Ctrl=Control

University of North Carolina, Chapel Hill UNCH-06-14VW Effect of obesity, AMPK activation and mTOR inhibiton on ovarian cancer tissue metabolism		Fold of Change			
		ANOVA Contrasts			
Sub Pathway	Biochemical Name	Non-obese:Met Non-obese:Ctrl	Obese:Met Obese:Ctrl	Non-obese: Everolimus Non-obese:Ctrl	Obese: Everolimus Obese:Ctrl
TCA Cycle	citrate	0.46	0.94	**0.29**	0.58
	alpha-ketoglutarate	1.23	1.53	0.91	1.25
	succinylcarnitine	**3.6**	2.44	2.13	2.3
	succinate	**3.29**	0.22	1.4	**0.27**
	fumarate	0.73	1.57	0.66	0.87
	malate	0.8	**1.4**	0.75	0.85

Table 4. The Effects of Metformin and Everolimus on the Biopterins in the Ovarian Tumors from Obese and Non-Obese Mice * Met= Metformin, Ctrl=Control

University of North Carolina, Chapel Hill UNCH-06-14VW Effect of obesity, AMPK activation and mTOR inhibiton on ovarian cancer tissue metabolism		Fold of Change			
		ANOVA Contrasts			
Sub Pathway	Biochemical Name	Non-obese:Met Non-obese:Ctrl	Obese:Met Obese:Ctrl	Non-obese: Everolimus Non-obese:Ctrl	Obese: Everolimus Obese:Ctrl
Tetrahydrobiopterin	biopterin	0.87	3.29	0.98	1.01
	dihydrobiopterin	0.75	8.53	1.83	1.63
Pterin Metabolism	pterin	0.43	2.65	0.9	1.92

Task 3 (Aim 3): Cross-species evaluation of differences between human and KpB mouse ovarian cancers in lean and obese states through genomics and metabolomics and primary culture.

We have collected ovarian tumors for primary culture from 10 OC patients to date. The tumors from these 10 patients were treated with metformin and everolimus in short term primary culture. Metformin (IC50 range 1-5 mM) and everolimus (IC50 range 68-250 nM) inhibited cell proliferation in all of the primary cultures to date, with parallel decreases in phosphorylation of S6, a downstream target of the mTOR pathway. We have not found any significant differences to response to metformin or everolimus in the ovarian tumors from obese versus non-obese women. We will continue to collect and treat primary cultures of OC tumors from obese and non-obese women in the upcoming report period.

Ovarian tumors from obese and non-obese OC patients have been identified in the LCCC tissue bank and are being sent to Metabolon for metabolomic analysis. The results from this analysis will be compared to that of the metabolomic analysis of ovarian tumors from obese and non-obese KpB mice and be reported in our next progress report.

To assess genomic differences between high grade serous ovarian tumors from obese versus non-obese women, we took advantage of the publically available gene expression analysis from The Cancer Genome Atlas (TCGA) database. From the TCGA database, we collected expression measurements for 12,042 genes from the platform (BI_HT_HG-U133A level 3 data) for differential gene expression analysis among human high grade, serous OC samples. The detailed information of the data processing, quality control and normalization can be found on the TCGA website. To identify significantly differentially expressed genes associated with BMI, we applied linear modeling for responses as gene expression and covariates as 5 principal components (PCs) (from gene expression data to control potential batch effects), clinical stage, grade, age, race, residual tumor and BMI status (0 if normal BMI < 25; 1 if overweight BMI ≥ 25). Appropriate false discovery rates (FDR) were controlled. With the obtained genes that were significantly associated with BMI status, we conducted functional clustering analysis on the website of The Database for Annotation, Visualization and Integrated Discovery (DAVID). In addition, we applied hierarchical clustering analysis to generate a representative heatmap. The Chi-square test was used to compare BMI among different clusters of samples. A comparison of the demographics between the OC tumors from normal weight (BM I< 25) and overweight/obese women (BMI ≥ 25) can be found in **Table 5**.

Table 5. Comparison of the demographics between the ovarian cancer tumors from normal weight and overweight/obese women.

	BMI < 25 (Normal Weight) (N=99)	**BMI ≥ 25 (Overweight/Obese) (N=138)**
Age (mean)	57.9	59.4
Race		
White	89 (90%)	125 (91%)
Black	5 (5%)	11 (8%)
Other	5 (5%)	2 (1%)
Grade		
2	11 (11%)	12 (9%)
3	88 (89%)	126 (91%)
Stage		
I/II	2 (2%)	4 (3%)
III/IV	97 (98%)	134 (97%)
Residual Disease		
Optimal	75 (76%)	99 (72%)
Suboptimal	24 (24%)	39 (28%)

347 genes were found to be significantly up- or down-regulated with BMI status (BMI < 25 versus BMI ≥ 25) among the serous ovarian tumors (q-value < 0.1), including metabolically relevant genes. Genes that were down-regulated included the prolactin receptor (3.6 fold) and apolipoprotein B mRNA editing enzyme (3.1 fold), among others. Genes that were up-regulated included mitogen-activated protein kinase 1 (3.3 fold), phospholipid scramblase 1 (3.3 fold), carnitine/acylcarnitine translocase (3.2 fold), low density lipoprotein receptor-related protein 8 (apolipoprotein e receptor) (3.7 fold), apolipoprotein L3 (3.7 fold), apolipoprotein L1 (3.8 fold), lipoyltransferase 1 (4.2 fold), apolipoprotein L6 (4.2 fold) and the c-myc binding protein (4.1 fold). Many of these genes were related to the apolipoprotein pathway, particularly apolipoprotein L related genes. Apolipoprotein L genes are members of the high density lipoprotein family and play a central role in cholesterol transport. Multiple genes involving the Ras oncogene family were up- and down-regulated when comparing normal weight versus overweight/obese women, including ras responsive element binding protein 1, RAB5C, RREB1, ras-related GTP binding C, PAP1A, RAB7A, RAB31, RAB5A, and ras homolog family/member A. DAVID functional annotation analysis revealed significant enrichment in "protein transport" (Adjusted p-value for Benjamini = 5.5E-5), "antigen processing and presentation of exogenous peptide antigen" (Adjusted p-value for Benjamini = 1.3E-3) and "pyrimidine ribonucleotide biosynthetic process" (Adjusted p-value for Benjamini = 3.6E-2) for these identified genes.

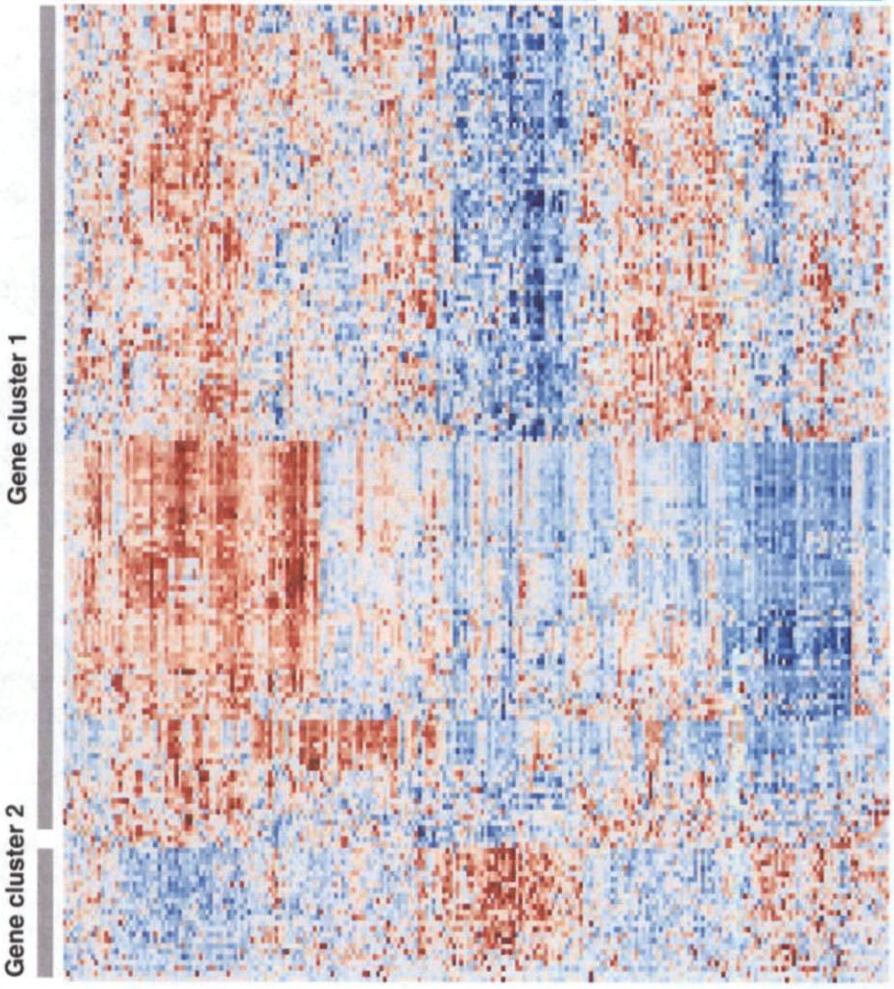


Figure 13. Genomic differences between ovarian tumors from normal weight versus overweight/obese women reveal alterations in metabolically relevant genes. Heat map representation of 264 genes significantly up- or down-regulated in ovarian tumors from normal weight versus overweight/obese women (FDR<0.05). Many metabolically relevant genes were upregulated in ovarian tumors from overweight/obese women such as mitogen-activated protein kinase 1, phospholipid scramblase 1, carnitine/acylcarnitine translocase, low density lipoprotein receptor-related protein 8 (apolipoprotein e receptor), apolipoprotein L3, apolipoprotein L1, lipoyltransferase 1, apolipoprotein L6 and the c-myc binding protein.

Initially, we used the 347 genes with q-value < 0.1 to generate a heatmap, but the results of the hierarchical cluster analysis on these samples did not group them with a significantly different BMI distribution. Alternatively, we used the 175 genes with q-value < 0.05 to generate a heatmap, which is presented in **Figure 13**, where the row signifies gene expression and the column is clustering according to BMI (BMI < 25 versus BMI ≥ 25). If we specified two groups to cut a tree resulting from the results of the hierarchical cluster analysis on the samples, the two clusters of samples had no statistically significant difference in the distribution of BMI. However, if we specified three groups to cut a tree resulting from the results of the hierarchical cluster analysis, there were two pairs of clusters of samples with a significantly different distribution of BMI. Specifically, the first

pair of clusters of samples (cluster 1 versus cluster 2) had sample proportions of subjects with BMI ≥ 25 (0.65, 0.33). For testing if the two proportions are significantly different, the obtained Chi-square statistics was 7.87, df = 1 and p-value = 0.005, suggesting that the two sample proportions are significantly different. The second pair of clusters of samples (cluster 3 versus cluster 2) had sample proportions of subjects with BMI ≥ 25 (0.61, 0.33). For testing if the two proportions are significantly different, the obtained Chi-square statistics was 11.36, df = 1 and p-value = 0.00075, suggesting that the two sample proportions are significantly different. In addition, there was significant difference in the proportions of women with a BMI ≥ 25 for cluster 1 and cluster 3 (0.65, 0.61). In summary, the analysis of the 175 gene set resulted in three sample clusters, with statistically significant differences in proportions of women with BMI ≥ 25 versus BMI <25 among these clusters. A summary of the genes in gene cluster 1 and 2 can be found in **Table 6**.

Table 6. Gene Clusters of the Ovarian Tumors from Normal Weight (BMI<25) and Overweight/Obese Women (BMI≥25).

	Gene Name	David Gene Name
Gene Cluster 1	FAP	fibroblast activation protein, alpha
	LAIR1	leukocyte-associated immunoglobulin-like receptor 1
	GPR65	G protein-coupled receptor 65
	RAB5C	RAB5C, member RAS oncogene family
	CTSK	cathepsin K
	RHOA	Ras homolog gene family, member A
	RAB5A	RAB5A, memver RAS oncogene family
	IL10RA	interleukin 10 recptor, alpha
	IL2RB	interluekin 2 receptor, beta
	LRP8	low density lipoprotein receptor-related protein 8
	APOL3	apolipoprotein L3
	APOL1	apolipoprotein L1
	CFLAR	CASP8 and FADD-like apoptosis regulator
	PLAU	plasminogen activator, urokinase
	RAB31	RAB31, member RAS oncogene family
	MYCBP	c-myc binding protein
	AAPOL6	apolipoprotein L6
	LIPT1	lipoyltransferase 1
	PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit
	PTPRC	protein tyrosine phosphotase, receptor type, C
	ETF1	eukaryotic translation termination factor 1
	EIF2B3	eukaryotic translational initiation factor 2B
	CASP1	caspase 1, apoptosis-related cysteine peptidase
Gene Cluster 2	IGSF3	immunoglobulin superfamily, member 3
	PRLR	prolactin receptor
	GRM4	glutamate receptor, metabotropic 4
	LY6G6E	lymphocyte antigen 6 complex, locus G6E
	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1
	ADRA1A	adrenergic, alpha-1A-, receptor

(4) KEY RESEARCH ACCOMPLISHMENTS

- Metformin and everolimus potentially drive glucose metabolism and uptake in OC cells, despite blunting proliferation.

- Metformin and everolimus inhibited proliferation in both cell lines under normal glucose conditions, through G1 cell cycle arrest.
- Metformin and everolimus were found to be more effective in the inhibition of proliferation under low versus high glucose concentrations, as also evidenced by enhanced G1 cell cycle arrest. In addition, metformin and everolimus were found to be more effective in the induction of apoptosis under low versus high glucose concentrations.
- As the concentration of glucose increased, phosphorylation of AMPK decreased and phosphorylation of S6 increased, suggesting increased proliferative capacity and hyperactivation of the mTOR pathway with high physiologic glucose.
- Metformin and everolimus both decreased glucose-stimulated ATP production, but had opposite effects on glucose uptake. Metfomin increased glucose uptake, and everolimus decreased glucose uptake.
- The obese state can promote tumor progression in the KpB mouse model of OC.
- Distinct metabolic and genomic differences were identified in ovarian tumors that arose in obese versus non-obese KpB mice, and many of these differences were related to metabolic relevant pathways.
- Metformin was more efficacious in the inhibition of tumor growth in our obese versus non-obese KpB mice, suggesting that obesity may be a biomarker for response to this agent. Everolimus had similar effects in the inhibition of tumor growth in obese and non-obese KpB mice.
- Metabolically relevant alterations in gene expression were found with increasing BMI among human serous OCs, using the TCGA database.

(5) CONCLUSION

Epithelial OC is one of the most lethal cancers among women in the United States, and minimal improvements in overall survival have been made in the past several decades. The lack of progress is largely attributable to late detection, drug resistance, and a high recurrence rate. Although chemotherapeutic agents that target specific cell signaling pathways have greatly expanded our profile of OC treatments, the challenge has been in identifying the patients that would most benefit from each of these diverse agents. To address these challenges, most previous research has focused on molecular alterations in the tumors derived from these patients. We postulate that focusing on the tumor alone may be too narrow a view and that the host environment, particularly the obese state, may play an equally important role in the selection of chemotherapeutic agents for effective treatment response. It is our hypothesis that obesity drives OC formation through alterations in metabolic pathways; and thus, inhibitors of one such pathway (mTOR) may be more efficacious in the obese versus non-obese state.

In order to answer this fundamental biological question regarding the role of the obese environment in oncogenesis, our approach is three fold using OC cell lines, a genetically engineered mouse model and patient samples. For each of these strategies, the metabolic state of obesity is being uniquely invoked and to test our hypothesis, two targeted mTOR pathway agents (metformin and everolimus) are being evaluated under obese versus non-obese conditions. To date, we have demonstrated that the obese state can promote tumor progression in the KpB mouse model of OC, as evidenced by a tripling of tumor size in obese versus non-obese mice. Diet-induced obesity was mimicked in the KpB mice through exposure to a HFD. The ovarian tumors that arose in the obese mice were genomically and metabolically different from those that arose in non-obese mice. Metformin, an AMPK activator, was found to be more efficacious in the inhibition of ovarian tumor growth in the obese versus non-obese KpB mice, suggesting that obesity may be a biomarker for response to this agent. In contrast, everolimus (RAD001), a mTOR inhibitor, was found to be equally efficacious in obese and non-obese mice. This difference in findings between these two targeted agents may be partially explained in that metformin increases glucose uptake and everolimus decreases glucose uptake as demonstrated in our *in vitro* studies (Aim 1) and by others (47). In addition, our preliminary metabolomic results of the effects of metformin versus everolimus in the ovarian tumors from obese and non-obese KpB mice indicate that metformin but not everolimus had differential effects depending on obesity status. Common toxicities associated with everolimus treatment include hyperglycemia and insulin resistance as opposed to metformin's favorable effects on improving insulin resistance and decreasing circulating glucose and insulin levels. Further metabolomic and genomic analysis of the ovarian tumors from metformin/control treated and everolimus/control treated obese and non-obese mice is underway to assess the potential differential effects of an AMPK activator versus a mTOR inhibitor on tumor growth.

For our *in vitro* studies, obesity/diabetes was mimicked by exposing OC cell lines to high versus low and normal glucose conditions. We had hypothesized that metformin and everolimus would be more efficacious in the setting of high versus low and normal glucose, but we found the opposite to be true. Metformin and everolimus were found to be more effective in the inhibition of proliferation under low versus high glucose concentrations, as also evidenced by enhanced G1 cell cycle arrest. In addition, metformin and everolimus were found to be more effective in the induction of apoptosis under low versus high glucose concentrations. We postulate that OC cells deprived of glucose may have blunted proliferative capacity, rendering them to be more susceptible to metformin and everolimus. Our studies also indicate that a high glucose environment enhances proliferative capacity. Metformin and everolimus were both found to decrease glycolysis in OC cells but had opposite effects on glucose uptake, with metformin increasing and everolimus decreasing glucose uptake. Further studies in the upcoming report period will focus on protein expression to evaluate the full effects of metformin and everolimus on downstream targets of the mTOR pathway under the varying glucose conditions.

We understand that the *in vitro* environment of high glucose exposure may not completely replicate that of the whole body *in vivo*; and thus, our animal and human studies are purposely meant to complement the cell culture work to better define the impact of obesity on OC development, progression and ultimately, treatment. In order to explore the impact of obesity on sensitivity to mTOR inhibitor and metformin therapy in the human disease, primary cultures of freshly isolated human OC cells derived from obese versus lean patients have been exposed to these agents. To date, we have not found any differences in response to metformin or everolimus between ovarian tumors from obese versus non-obese women in primary culture.

Alterations in gene expression were found with elevated BMI in serous OC tumors in the TCGA database. Many of the genes with differential expression were related to lipid metabolism and the apolipoprotein pathway, which is important in triglyceride and cholesterol transport, and this pathway will be a focus of our future work. Our findings demonstrate that obesity may contribute to OC pathogenesis through the differential expression of metabolically relevant genes. Metabolomic analysis of ovarian tumors from obese and non-obese women is underway, and these results will be correlated to the gene expression profiling results as well as parallel studies in the KpB mouse model.

Findings from this proposal should determine if obesity-driven OCs are biologically divergent and whether these inherent differences play a role in sensitivity to chemotherapeutic agents, and we have already provided evidence to support this hypothesis. This work may ultimately lead to the individualization of OC treatment based on both tumor biology and the metabolic composition of the patient. This study will initially investigate this concept for the mTOR inhibitor everolimus and metformin in OC, but may ultimately translate to other emerging therapies targeted to this pathway or others identified in this proposal. Future clinical trials of targeted therapies would have to be structured to address the host/tumor interaction, and we would propose that stratifying for obesity status may be an initial approach in this pursuit. To our knowledge, no cancer chemotherapeutic clinical trial has addressed obesity status as a contributing factor to therapeutic success. Lastly, this knowledge of the impact of obesity on response to targeted therapies would be important not just for OC but for all cancers where obesity is associated with increased risk and worse outcomes, such as endometrial, breast and colon cancer among others.

(6) PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Abstracts presented:

(1) Zhou, C, Zhong, Y, Du, X, Makowski, L, Jia, W and Bae-Jump, VL, Diet-induced obesity increases tumor aggressiveness in a genetically engineered mouse model of serous ovarian cancer, Oral Presentation by Dr. Bae-Jump at the 2013 Annual Meeting of the Society of Gynecologic Oncology. *

(2) Jackson, A, Zhong, Y, Zhou, C, Kilgore, J, Makowski, L, Gehri,g P, Bae-Jump, V. Metformin had increased efficacy under obese conditions in a novel genetically engineered mouse model of serous ovarian cancer. 45th Annual Meeting of the Society of Gynecologic Oncology, March 2014, Tampa, Florida.

Manuscripts accepted:

(1) Makowski, L, Zhou, C, Zhong, Y, Kuan, PF, Fan, Sampey, BP, Difurio, M and Bae-Jump, VL#. Obesity increases tumor aggressiveness in a genetically engineered mouse model of serous ovarian cancer, Gynecol Oncol, 2014, Apr;133(1):90-7. PMID: 24680597

(7) INVENTIONS, PATENTS AND LICENSES: NONE

(8) REPORTABLE OUTCOMES: NONE

(9) OTHER ACHIEVEMENTS

Grants submitted:
(1) 1R01CA194098-01
Obesity, Cation-Selective Transporters and Metformin in Endometrial Cancer
$1,250,000 (total direct costs), $250,000 (annual direct cost for year one)
Co-Principal Investigator: Bae-Jump
15% effort (1.8 calendar)
Mounting epidemiological and preclinical data suggest that metformin may be efficacious in endometrial cancer. However, two important questions that need to be addressed are: **(1)** Will metformin be universally effective in endometrial cancer or be more efficacious in the obese/insulin-resistant patient population? and **(2)** What role do transporters play in metformin uptake and action in the malignant endometrium? These fundamental questions will be explored in endometrial cancer, a disease driven by obesity and insulin resistance, using endometrial cancer mouse models and phase 0 and phase 2/3 clinical trials in endometrial cancer patients.

(2) RSG CCE-127311
American Cancer Society/Research Scholar Grant
Obesity, Cation-Selective Transporters and Metformin in Endometrial Cancer
$791,729 (total direct costs), $177,376 (annual direct cost for year one)
Principal Investigator: Bae-Jump
10% effort (1.2 calendar)
Mounting epidemiological and preclinical data suggest that metformin may be efficacious in endometrial cancer. However, two important questions that need to be addressed are: **(1)** Will metformin be universally effective in endometrial cancer or be more efficacious in the obese/insulin-resistant patient population? and **(2)** What role do transporters play in metformin uptake and action in the malignant endometrium? These fundamental questions will be explored in endometrial cancer, a disease driven by obesity and insulin resistance, using endometrial cancer mouse models and phase 0 and phase 2/3 clinical trials in endometrial cancer patients.

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Obesity increases tumor aggressiveness in a genetically engineered mouse model of serous ovarian cancer☆

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HIGHLIGHTS

- Obesity promotes tumor progression in the KpB mouse model of serous ovarian cancer.
- Gene expression and metabolomic profiling indicated significant differences between ovarian tumors from obese versus non-obese mice, including metabolically relevant pathways.

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ABSTRACT

Objectives. Obesity is associated with increased risk and worse outcomes for ovarian cancer. Thus, we examined the effects of obesity on ovarian cancer progression in a genetically engineered mouse model of serous ovarian cancer.

Methods. We utilized a unique serous ovarian cancer mouse model that specifically deletes the tumor suppressor genes, Brca1 and p53, and inactivates the retinoblastoma (Rb) proteins in adult ovarian surface epithelial cells, via injection of an adenoviral vector expressing Cre (AdCre) into the ovarian bursa cavity of adult female mice (KpB mouse model). KpB mice were subjected to a 60% calories-derived from fat in a high fat diet (HFD) versus 10% calories from fat in a low fat diet (LFD) to mimic diet-induced obesity. Tumors were isolated at 6 months after AdCre injection and evaluated histologically. Untargeted metabolomic and gene expression profiling was performed to assess differences in the ovarian tumors from obese versus non-obese KpB mice.

Results. At sacrifice, mice on the HFD (obese) were twice the weight of mice on the LFD (non-obese) (51 g versus 31 g, $p = 0.0003$). Ovarian tumors were significantly larger in the obese versus non-obese mice (3.7 cm^2 versus 1.2 cm^2, $p = 0.0065$). Gene expression and metabolomic profiling indicated statistically significant differences between the ovarian tumors from the obese versus non-obese mice, including metabolically relevant pathways.

Introduction

Obesity has been linked to increased risk of many cancers, including breast, colon, endometrial, among others [1]. Currently, new cancer cases are in the order of 1.5 million with half a million cancer deaths per year, and nearly one in five are due to obesity [1,2]. It is postulated that hyperglycemia and hyperinsulinemia resulting from overnutrition in obese patients provide abundant nutrients and growth factors to cancer cells, resulting in the ideal environment for tumor initiation and promotion [3]. Chronic inflammation and immunosuppression are also thought to be a link between obesity and cancer [3].

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Epithelial ovarian cancer (OC) is one of the most deadly cancers with an overall 5-year survival of only 30–40%. Increasing evidence suggests that obesity is a significant risk factor for OC and associated with worse outcomes for this disease [1,4–20]. Given the overall poor prognosis of OC and the rising rate of obesity, it is imperative to investigate obesity as a potential modifiable risk factor that may reverse risk and lead to the prevention and improvement of outcomes for OC. We hypothesize that the metabolic consequences of obesity may play a contributing role in the pathogenesis of OC and may lead to biologically and phenotypically different cancers than those that arise in normal weight women, possibly necessitating distinct treatment strategies. Herein, we assessed the impact of obesity on OC development and progression in a genetically engineered mouse model of serous OC and comprehensively interrogated the obesity-induced carcinogenesis signature through genomic and metabolomic analysis.

Materials and methods

Obesity and the K18-gT$^{+/-}_{121}$;p53$^{fl/fl}$;Brca1$^{fl/fl}$ mouse model

The K18-gT$^{+/-}_{121}$;p53$^{fl/fl}$;Brca1$^{fl/fl}$ (KpB) mouse model (Terry Van Dyke, PhD, NIH) is a unique serous OC mouse model, wherein the tumor suppressor genes, Brca1 and p53 are specifically and somatically deleted and the retinoblastoma (Rb) proteins are inactivated in the adult ovarian surface epithelium [21]. Inactivation of all 3 Rb proteins by T_{121} (a fragment of the SV40 large T antigen) is driven by the keratin 18 (K18) promoter [21]. Expression of the T_{121} transgene and knockout of p53 and Brca1 are conditional and only activated via injection of an adenoviral vector expressing Cre (AdCre) into the ovarian bursa cavity of adult female mice. At approximately 6 months after AdCre injection, tumors develop in the affected ovary, while the un-injected ovary remains normal.

All experimental animals were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) and the NIH guide for the Care and Use of Laboratory Animals. Recombinant adenovirus Ad5-CMV-Cre (AdCre) was purchased from the University of Iowa Transfer Vector Core at a titer of 10^{11}–10^{12} infectious particles/ml. To maximize weight gain, mice were provided a high-fat diet (HFD, obese group) (60% kcal from fat, Research Diets, New Brunswick, NJ) and control mice (non-obese group) were provided a low-fat diet (LFD) (10% kcal from fat, Research Diets, New Brunswick, NJ) ad libitum, beginning at 6 weeks of age. AdCre injection occurred at 8 weeks to induce OC 6 months later (at 8 months of age) [21]. Thirty-six hours following superovulation, the mice were anesthetized, and a single 1 cm incision was made on the dorsal surface of each mouse. The AdCre was then injected via a needle introduced into the oviduct near the infundibulum and into the ovarian bursa, and the incision was closed. All mice were sacrificed at 8 months of age.

The primary outcome comparison between non-obese and obese mice was the response of tumor growth to the obesity exposure. This was assessed via direct measurement of the tumor at the time of sacrifice. At the time of sacrifice, the ovarian tumors were harvested, wet tumor weights recorded, and tissue was snap frozen in liquid nitrogen for later harvest of mRNA for microarray analysis and metabolites for metabolomic analysis.

Body weight & composition

Prior to starting mice on diet and weekly until sacrifice, body weight was measured. Body composition, including lean mass, fat mass, free water content and total water content, of non-anesthetized mice was also measured at pre- and post-diet exposures using the EchoMRI-100 quantitative magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, TX).

Blood glucose

Random blood glucose was measured prior to start of diet and at sacrifice using a Bayer Contour Blood Glucose Monitor (Bayer HealthCare LLC, Tarrytown, NY).

mRNA isolation

Approximately 25–50 mg of frozen OC tissue in small fragments was homogenized in RLT lysis buffer. Total RNA was isolated using the RNeasy mini kit and QIAshredder kit (Qiagen Inc., Mississauga, ON) following the manufacturer's instructions. RNA quantity and quality were analyzed by Nanodrop (Thermoscientific, Wilmington, DE).

Gene expression profiling

Microarrays were performed on ovarian tumors from non-obese and obese mice (N = 5/group) using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. These samples were processed in the Lineberger Comprehensive Cancer Center Genomics Core Facility. The image files were analyzed with GenePix Pro 4.1 and pre-processed via the UNC-Chapel Hill Microarray Database (https://genome.unc.edu) where a Lowess normalization procedure was performed to adjust for Cy3 and Cy5 channel biases [22]. In addition, probes with missing values in 3 or more samples in each of the obese and non-obese groups were removed. Two-class SAM (Significance Analysis of Microarrays, http://www-stat.stanford.edu/~tibs/SAM/) was performed to identify significantly differentially expressed genes using FDR < 0.2. EASE (Expression Analysis Systematic Explorer, http://david.niaid.nih.gov/david/ease.htm) analysis was used to interpret and identify biological themes (gene ontology categories) overrepresented in the gene list obtained from SAM results. The EASE Score was used as statistical measure of overrepresentation of a biological theme. Specifically, the EASE Score is a jackknifed one-tailed Fisher's exact probability which is calculated by removing one gene within the given category from the list and penalizes the statistical significance of categories supported by fewer genes; thus is a more robust measure than the Fisher's exact probability [23].

Metabolomic profiling

Gas chromatography time-of-flight mass spectrometry (GC-TOFMS, Leco Corporation, St Joseph, MI) and liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOFMS, Agilent Corporation, Santa Clara, CA) were used to analyze tumors from non-obese and obese mice (N = 5/group). Metabolite extraction followed previous publication with minor revision through the UNC/Nutrition Obesity Research Center (NORC) Core facility [24]. Briefly, 50 mg samples were extracted with 0.5 ml of methanol:chloroform:water = 3:1:1 (v:v:v) with homogenization for 3 min using 1-mm inner diameter balls in a Bullet Blender (Next Advance, Averill Park, NY). Two aliquots of 150 μl of supernatant were used for GC-TOFMS and LC-TOFMS analysis, separately. After removal of the extra supernatant, the remainder was extracted with 500 μl of methanol. Two aliquots of 150 μl of supernatant were combined into the tube containing first step extraction for GC and LC-TOFMS analysis, separately. Metabolite annotation was performed by comparing the mass spectrum and retention time to an in-house library and NIST library (GC-TOMFS) or HMDB (LC-TOFMS) [25,26].

Statistical methods

Unpaired Student's *t*-test was used to determine statistical difference between non-obese and obese treatment groups using STATA software (College Station, TX). A p-value <0.05 was considered significant. For metabolomics, after normalization to the internal standard and sample weight, the data set was imported into SIMCA-p software

(Umeå, Sweden) for multivariate analysis. Principle component analysis (PCA) was first performed to check the outliers and the separation tendency (data not shown). A supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) analysis was then performed. Differentiating metabolites were selected with the criteria of the variable importance in the projection (VIP) value > 1 and p value (Student's *t* test) lower than 0.05.

Results

Obesity drove significant tumor progression in KpB mice

KpB mice were subjected to 60% calories-derived from fat in a high fat diet (HFD) versus 10% calories from fat in a low fat diet (LFD) to induce diet-induced obesity (N = 14/group) starting at 6 weeks of age and until sacrifice. After 8 months of exposure to the HFD or LFD, obese mice weighed significantly greater than non-obese mice ($p = 0.003$, Table 1). There was no effect of HFD on non-fasted blood glucose levels in KpB mice over the course of the diet (Table 1). Body composition was significantly altered in obese KpB mice compared to non-obese controls. Percent body fat was six-fold greater in obese mice (Table 1, $p = 0.0001$), while percent lean mass increased by 25% ($p = 0.0006$, Table 1). The ovarian tumors were tripled in size in the obese mice as compared to non-obese mice (mean size of 3.7 cm^2 versus 1.2 cm^2, Fig. 1, $p = 0.0065$).

Obesity induces genomic differences between obese and non-obese ovarian tumors

439 genes were found to be significantly up-regulated (417 genes) or down-regulated (22 genes) in the ovarian tumors from obese KpB mice versus non-obese mice (FDR < 0.2, Supplemental Table 1). Fig. 2 is a heat map of 131 genes up- and down-regulated at a FDR < 0.1. Metabolically relevant genes were significantly upregulated in the ovarian tumors from the obese versus non-obese mice, such as lipocalin (2.7 fold), fatty acid amide hydrolase (2.7 fold), fatty acid 2-hydroxylase (2.2 fold), glycerol-3-phosphate acyltransferase (1.5 fold), protein phosphatase (1.2 fold), AMP deaminase 3 (1.6 fold), and protein kinase C (1.7 fold) (Supplemental Table 1). Arginase 1 was the most upregulated gene (7.3 fold) and plays a role in the urea cycle, tissue remodeling and inflammation. Other upregulated genes identified in the ovarian tumors from the obese mice were related to cell adhesion, including neurotrimin (2.2 fold) and desmoglein 1-alpha (2.0 fold). Increased expression of histone 1 (2.3 fold), endothelin-1 (5.8 fold), ectonucleoside triphosphate diphosphohydrolase (3 fold) and serotonin transporter solute carrier family 6 member 4 (Slc6a4) (5.4 fold) were also associated with obesity in the KpB mouse model. Significantly downregulated genes with obesity included spermidine synthase and thrombospondin 4.

In the ovarian tumors from the obese versus non-obese mice, EASE over-representation analysis revealed significant enrichment in "phospholipid binding" (EASE score of 0.008), "regulation of apoptosis" (EASE score of 0.014), "lipid binding" (EASE score of 0.015), "endopeptidase activity" (EASE score of 0.03) and "cell–cell signaling" (EASE score of 0.44) for those identified genes.

Table 1
Diet-induced metabolic characteristics in non-obese and obese KpB mice.

	Non-obese	Obese	p-Value
Weight (g)	31.14 ± 5.26	50.71 ± 16.73	p = 0.0003
Glucose (mg/dl)	186.81 ± 26.99	214.38 ± 58.11	p = 0.053
% fat	3.28 ± 1.51	19.58 ± 7.88	p = 0.00001
% lean	22.89 ± 2.11	28.66 ± 5.24	p = 0.0006

N = 14 mice per group. Mean ± SD. % fat or % lean = each mass / total body mass as measured by MRI.

Metabolic differences between ovarian tumors from obese and non-obese KpB mice

Principle component analysis defined a clear separation between obese and non-obese samples (Fig. 3, 3 components, R2X = 0.563, R2Ycum = 0.95, Q2cum = 0.411). Differentiating metabolites were selected with the criteria of the variable importance in the projection (VIP) value > 1 and p value (Student's *t* test) lower than 0.05. Twenty metabolites were identified using this criterion, all of which were upregulated in the ovarian tumors of the non-obese versus obese KpB mice (Table 2).

Metabolites involved in inflammatory signaling and protein/collagen metabolism were down-regulated in the ovarian tumors of obese mice as compared to non-obese mice, including arginine ($p = 0.0268$), N-glycylproline ($p = 0.0043$) and 3-amino-2-piperidone ($p = 0.0099$). Components and markers of oxidative stress were also downregulated in the tumors from obese mice: glutathione ($p = 0.0313$), oxidized glutathione ($p = 0.0047$), gluconolactone ($p = 0.0311$) and 8-hydroxydeoxyguanosine ($p = 0.0230$). Lower levels of nucleotides (i.e. cytidine ($p = 0.0122$ and $p = 0.0424$), cytosine ($p = 0.0158$), guanosine diphosphate (GDP, $p = 0.0404$)) and adenosine monophosphate (AMP, $p = 0.0257$) were detected with obesity. The serotonin metabolite, 5-hydroxyindoleacetic acid (5HIAA, $p = 0.0498$), and the catecholamine metabolites, vanillactic acid ($p = 0.0079$) and phenylethanolamine ($p = 0.0446$), were found to be lower in the ovarian tumors of obese versus non-obese mice. Glutamate ($p = .0318$), N-acetylaspartic acid ($p = 0.0059$) and succinic acid ($p = 0.0465$) are involved in energy metabolism, and were decreased in the ovarian tumors of obese KpB mice. LysoPC(16:1(9Z)) ($p = 0.0205$), a lysophospholipid, was also lower in the ovarian tumors from obese animals.

Discussion

Recent evidence suggests that obesity may be a significant risk factor and associated with worse outcomes for OC [1,4–20]. Therefore, a metabolic approach to the diagnosis and treatment of OC may provide a novel strategy to improve outcomes for this invariably lethal disease. Hence, we induced obesity in the KpB mouse, a faithful murine model of serous OC, to ask if obesity alters tumorigenesis. KpB mice fed a HFD had significant increases in their body weight and fat mass compared to mice fed a LFD. Herein, we report that obesity promoted tumor progression in the KpB mouse model of OC with a tripling of ovarian tumor size. Obesity has been associated with more rapid tumor growth in animal models of other cancer types, such as breast, colon and lung cancer [27,28], but this is the first study to demonstrate this for OC.

Genomic and metabolomic analyses were utilized to identify obesity-induced alterations in tumors with the intention of identifying significant pathways or biomarkers to aid in explaining why obese mice developed larger, more aggressive tumors. The metabolically relevant genes, lipocalin, ectonucleoside triphosphate diphosphohydrolase and fatty acid amide hydrolase, were upregulated in the ovarian tumors from the obese versus non-obese mice. Lipocalin, particularly lipocalin 2, has been previously found to be upregulated in number of different cancers, including OCs [29,30]. The primary function of lipocalin is the transport of small ligands such as steroids, bilins, retinoids and lipids. In addition to its role in lipid transport, lipocalin has also been implicated in the inflammatory response. Another gene significantly upregulated was ectonucleoside triphosphate diphosphohydrolase, which is involved in the extracellular hydrolysis of ATP to generate adenosine, which signals through G-protein coupled receptors and regulates metabolic pathways and inflammation. Chronic inflammation is well known to play a role in obesity-driven cancers which could also explain the increased expression of both lipocalin and ectonucleoside triphosphate diphosphohydrolase in the ovarian tumors of obese KpB mice.

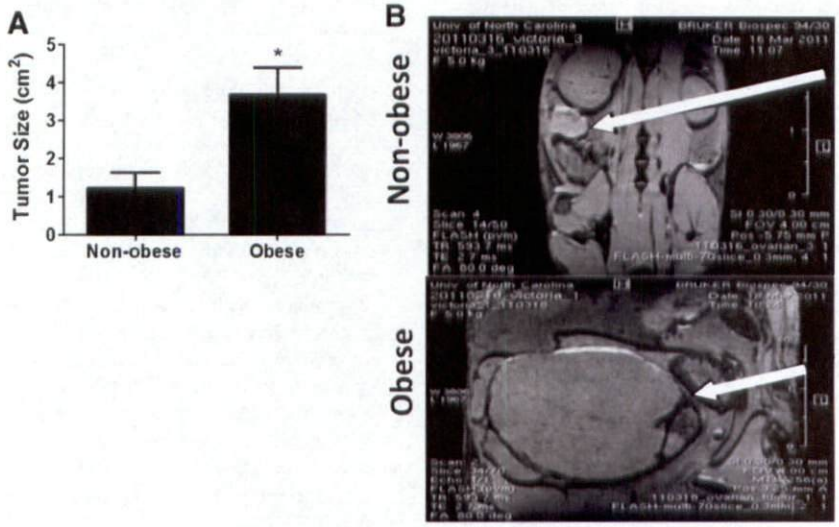

Fig. 1. Obesity increases tumor size in KpB mice. KpB mice were fed low fat or high fat diets to induce obesity for 6 months during tumorigenesis. (A) Comparison of tumor size from non-obese and obese mice (N = 14). These mice were sacrificed 6 months after ovarian tumor induction via injection of AdCre into the ovarian bursa cavity. For the calculation of tumor size, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined and multiplied (m^2). *p = 0.0065. (B) MRI images of tumors (arrow) from non-obese (top image) and obese (bottom image) mice demonstrate representative tumors.

Fatty acid amide hydrolase (FAAH) is a serine hydrolase that metabolizes N-acylethanolamines (i.e. N-arachidonoylethanolamine, N-oleoylethanolamine and N-palmitoylethanolamine), also known as endocannabinoids, to fatty acids plus ethanolamine. The endocannabinoid system is thought to be important in the regulation of cancer cell apoptosis, proliferation, migration, adhesion and invasion. Increased expression of the cannabinoid receptors (CB1R and CB2R) and FAAH has been documented in prostate and breast cancer and has been associated with worse outcomes [31]. FAAH inhibitors are under development for the treatment of pain and inflammation [31], but may also be useful in cancer. Our data suggests that FAAH inhibitors might be a potential targeted agent for obesity-driven cancers.

Other unique, metabolically relevant genes that were associated with obesity and OC development in the KpB mouse model included fatty acid 2-hydroxylase, glycerol-3-phosphate acyltransferase, protein phosphatase, protein kinase C and AMP deaminase. Fatty acid 2-hydroxylase (FA2H) catalyzes the synthesis of 2-hydroxysphingolipids, a subset of sphingolipids that contain 2-hydroxy fatty acids. FA2H is thought to be involved in the cell differentiation of Schwann cells, keratinocytes and adipocytes. Glycerol-3-phosphate acyltransferase is an enzyme that participates in glycerolipid metabolism and glycerophospholipid metabolism. Protein phosphatases are essential to protein phosphorylation, an important form of reversible protein posttranslational modification involved in cell signaling cascades. The protein kinase C (PKC) family represents a number of protein kinase enzymes that are involved in regulating the function of other proteins through the phosphorylation

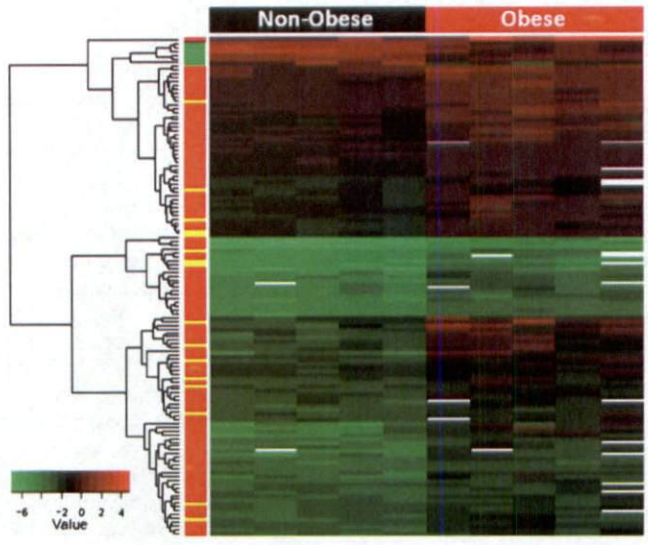

Fig. 2. Genomic differences between ovarian tumors from obese versus non-obese KpB mice reveal alterations in metabolically relevant genes. Heat map representation of 131 genes found to be significantly up- or down-regulated in the ovarian tumors from the obese versus non-obese KpB mice (FDR < 0.1). Many metabolically relevant genes, such as lipocalin, fatty acid amide hydrolase, ectonucleoside triphosphate diphosphohydrolase, fatty acid 2-hydroxylase, glycerol-3-phosphate acyltransferase, protein phosphatase, protein kinase C and AMP deaminase 3, were upregulated in obese tumors.

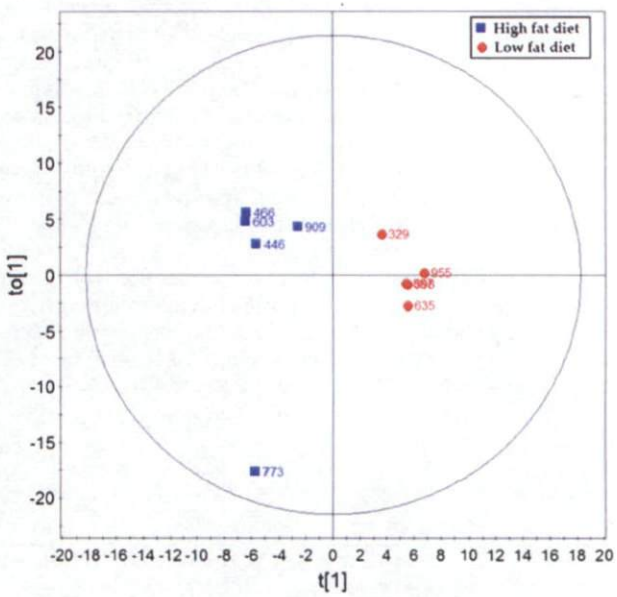

Fig. 3. Several metabolites define a clear separation using principal component analysis between the ovarian tumors in the non-obese group and obese group. PLS-DA scores plot of the ovarian tumors in the non-obese group (low fat diet) and obese (high fat diet) group.

Table 2
Metabolic alterations in tumors from non-obese and obese KpB mice.

Compound name	VIP[a]	p[b]	Fold change (non-obese/obese)[c]	Analysis method	Identification method[d]
N-glycylproline	2.27	0.0043	1.95	LC-ES+	Std
Oxidized glutathione	2.25	0.0047	3.45	LC-ES+	Std
N-acetylaspartic acid	2.22	0.0059	2.31	LC-ES−	HMDB
Vanillactic acid	2.17	0.0079	2.23	LC-ES+	HMDB
3-Amino-2-piperidone	2.14	0.0099	1.75	GCTOF	NIST
Cytidine	2.10	0.0122	4.52	LC-ES+	Std
Cytosine	2.05	0.0158	4.11	LC-ES+	Std
LysoPC(16:1(9Z))	1.99	0.0205	1.83	LC-ES+	HMDB
8-Hydroxy-deoxyguanosine	1.97	0.0230	2.45	LC-ES+	HMDB
Adenosine monophosphate	1.94	0.0257	1.61	LC-ES−	HMDB
Arginine	1.93	0.0268	1.93	LC-ES+	Std
Gluconolactone	1.89	0.0311	2.97	LC-ES+	Std
Glutathione	1.89	0.0313	3.10	LC-ES+	Std
Glutamate	1.89	0.0318	1.52	GCTOF	Std
Guanosine diphosphate	1.82	0.0404	2.39	LC-ES−	HMDB
Cytidine	1.81	0.0424	4.97	GCTOF	NIST
Inodxyl glucuronide	1.80	0.0439	3.05	LC-ES+	HMDB
Phenylethanolamine	1.80	0.0446	1.69	GCTOF	NIST
Succinic acid	1.78	0.0465	1.90	GCTOF	Std
5-Hydroxyindoleacetic acid	1.76	0.0498	1.85	LC-ES+	HMDB

[a] Variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0.
[b] p value was calculated from Student's *t* test.
[c] Fold change with a value larger than 1 indicates a relatively higher concentration in tumors from non-obese (low fat diet-fed) KpB mice, while a value less than 1 means a relatively lower concentration as compared to tumors from obese (high fat diet-fed) KpB mice.
[d] The metabolites were identified by in-house library (Std), NIST library (NIST) or HMBD database (HMDB).

of hydroxyl groups of serine and threonine amino acid residues on these proteins. The PKC family of enzymes has been implicated in the regulation of signal transduction, cell proliferation, metabolism and differentiation through its effects on regulation of the cell cycle. PKC inhibitors are already being evaluated in clinical trials for a variety of different cancers, including OC [32]. AMP deaminase 3 is a highly regulated enzyme that catalyzes the hydrolytic deamination of adenosine monophosphate to inosine monophosphate, a branch point in the adenylate catabolic pathway. AMP deaminase 3 is thought to be a potent regulator of energy metabolism in cells. Increased expression of AMP deaminases has been documented in hepatocellular carcinomas [33] but has not been explored in OC.

Although many metabolically relevant genes were found to be associated with obesity-driven cancers in the KpB mouse model, other upregulated genes and pathways were identified. This included genes related to cell adhesion, including neurotrimin and desmoglein 1-alpha. Expression of neurotrimin and desmoglein 1-alpha has not been previously documented in OCs. Increased expression of histone 1 in the ovarian tumors was also associated with obesity in the KpB mice. Histones are the chief protein component of chromatin and are critical for gene regulation. Endothelin-1 (ET-1) is a highly potent vasoconstrictive peptide and was found to be upregulated 5.8 fold in the ovarian tumors from obese mice. Overexpression of ET-1 has been implicated in the epithelial–mesenchymal transition, a mechanism by which transformed epithelial cells acquire the ability to proliferate, invade, resist apoptosis and metastasize [34]. In chemoresistant ovarian cancer cells, ET-1 has been found to be upregulated, leading to enhanced signaling through the MAPK and mTOR/Akt pathway, increased cell proliferation and reduced sensitivity to cisplatin and paclitaxel [35]. Endothelin receptor antagonists are being developed as potential chemotherapeutic agents for cancer [34]. In the ovarian tumors from the obese versus non-obese mice, DAVID functional annotation analysis revealed significant enrichment in "phospholipid binding", "regulation of apoptosis", "lipid binding", "endopeptidase activity" and "cell–cell signaling". Thus, the increase in aggressiveness, as manifested by a tripling of tumor size, in the obese KpB mice was accompanied by upregulation of genes involved in metabolic, apoptotic and cell signaling pathways.

Metabolic analysis revealed that 20 metabolites were identified as significantly regulated. In general, metabolomic analysis revealed that multiple metabolites contributed to separation of non-obese and obese mice with each metabolite being down-regulated in tumors derived from obese mice. Arginase 1 was the most up-regulated gene in obese tumors, which explains the lower detection of arginine concentrations. Catabolic disease states such as sepsis, injury and cancer cause an increase in arginine utilization, which can exceed normal body production, leading to arginine depletion. Arginase 1 converts L-arginine into L-ornithine and urea. Nitric oxide (NO) synthase and arginase compete for the same substrate (L-arginine); hence high arginase activity will blunt NO production, limiting potential pro-inflammatory responses necessary in tumoricidal immune responses. Indeed, arginase 1 is a marker of the M2, alternatively activated, macrophage that is often associated with more aggressive tumors [36]. Arginase also drives polyamine (such as spermidine) synthesis necessary for proliferation. Spermidine synthase in spermidine synthesis was a down-regulated gene in tumors from obese animals, perhaps in a negative feedback mechanism due to elevated delivery of ornithine generated by arginase 1 (30% lower levels of spermidine were detected in ovarian tumors of obese mice but this did not reach statistical significance). Ornithine can also be converted to the delta-lactam 3-amino-2-piperidine, and this was significantly blunted in tumors from obese mice. Finally, arginase generates ornithine which is used to generate proline (necessary for collagen synthesis) and glutamate/glutamine. Glutamate was found at lower levels suggesting that arginase was directing ornithine production to modulate collagen synthesis in tumors derived from obese mice. AMP and arginine both activate AMP kinase (AMPK) which stimulates substrate metabolism, while arginine can also activate mTOR [37,38]. Decreased concentrations of both AMP and arginine in the ovarian tumors from obese versus non-obese mice may be a reflection of increased turnover of these metabolites in the rapidly growing tumors in the obese mice, and potential regulation of substrate metabolism.

N-glycylproline, which had the highest VIP contributing to separation between non-obese and obese tumors, was significantly lower in obese tumors relative to non-obese tumors in KpB mice (Table 2, $p = 0.0043$). N-glycylproline is an end product of collagen metabolism, but may be recycled into collagen synthesis, and this suggests a potential difference in tissue remodeling between non-obese and obese mice. Overall, Fig. 4 depicts metabolites and genes related to arginine/polyamine/collagen/glutamine metabolism that were decreased in the ovarian tumors from obese mice, suggesting that diet-induced alterations in the stromal components and

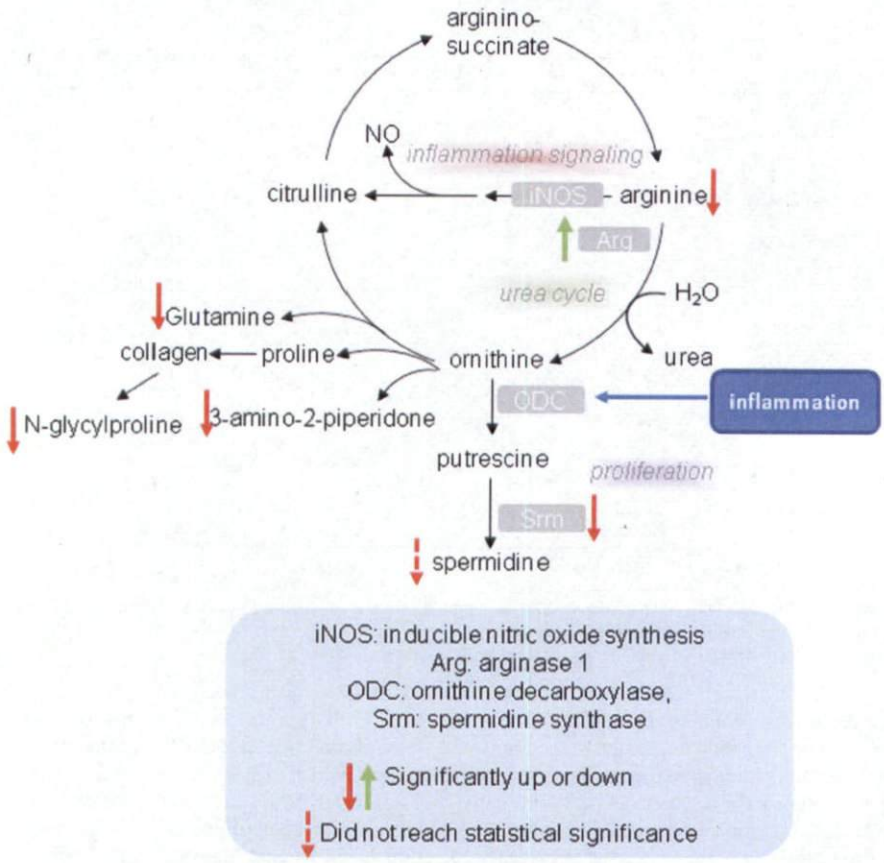

Fig. 4. Obesity-induced alterations in arginine/polyamine/collagen/glutamine metabolism. Metabolomic profiling of ovarian tumors from obese and non-obese KpB mice revealed significant decreases in a number of metabolites related to arginine/polyamine/collagen/glutamine metabolism, suggesting that diet-induced alterations in the stromal components and extracellular matrix are associated with greater growth of the ovarian tumors in obese animals.

extracellular matrix are associated with greater growth of the ovarian tumors in obese animals.

Although glutathione disulfide (GSSG) and glutathione (GSH) were significantly regulated by diet, the ratio of the two (as an indicator of oxidative stress) was not significantly different between lean (0.5 −/+ 0.048) and obese (0.45 −/+ 0.284) tumors, suggesting that there was no active oxidative stress. However, a more stable marker of oxidative stress-induced DNA modification, 8-hydroxydeoxyguanosine, was detected at significantly lower concentrations in obese versus non-obese tumors. Lower concentrations of gluconolactone, an oxidized derivative of glucose, were also found in tumors from obese animals relative to lean, providing further evidence of changes in reduction–oxidation status between the ovarian tumors in the non-obese versus obese group. In sum, in ovarian tumors in obese KpB mice, there appears to be less DNA modification and markers of oxidized metabolites due to oxidative stress, suggesting that oxidative stress is not a major driver of obesity-driven tumorigenesis in the KpB mice or that compensatory mechanisms exist. Alternatively, it could be that the greater growth of ovarian tumors in the obese animals was driven by inflammatory cytokines produced in adipose tissue and distributed to the tumor through the circulation.

Lower concentrations of nucleotides (i.e. cytidine, cytosine, guanosine diphosphate (GDP), adenosine monophosphate (AMP)) may be reflective of increased cell turnover and alterations in utilization and production of these building blocks in the ovarian tumors from obese versus non-obese mice. We postulate that the observed heightened proliferation in the ovarian tumors from the obese versus non-obese mice, as evidenced by a tripling of tumor size, may result in the increased consumption of nucleotides. In the genomic analysis, we also found a 3-fold increase in ectonucleoside triphosphate diphosphohydrolase. This enzyme catalyzes the breakdown of multi-phosphated nucleotides (i.e. ATP, ADP, etc.) and removes free nucleotides and upstream compounds like AMP and GDP, all of which were significantly decreased in the ovarian tumors from the obese mice. In addition, low AMP detected in the ovarian tumors from obese mice suggests possible elevations in anabolic, ATP-burning processes such as lipid synthesis as well as protein, RNA and DNA synthesis.

5-Hydroxyindoleacetic acid (5HIAA) was significantly lower in obese versus non-obese tumors. 5HIAA is a breakdown product of serotonin. Interestingly, the serotonin transporter solute carrier family 6 member 4 (Slc6a4) was upregulated 5.4 fold by obesity. In addition to its function as a neurotransmitter in the central nervous system, increasing evidence suggests that peripheral serotonin may have pro-proliferative and anti-apoptotic effects and act as a mitogen in cancer cells [39,40]; hence, the obesity-mediated regulation of serotonin is of interest.

The catecholamine metabolites, vanillactic acid and phenylethanolamine, were lower in ovarian tumors derived from obese animals. Catecholamines, including epinephrine and norephinephrine, are known to regulate lipolysis [41]. Several studies report that catecholamine responses are blunted in obese versus non-obese individuals at rest and in response to physical activity, suggestive of decreased sympathetic nervous system activity [41]. A decrease in the catecholamine response in the obese mice could lead to reductions in lipolysis and an increase in fat stores that could be advantageous for cancer cell growth.

Succinic acid and glutamate were also significantly decreased by obesity in tumors (Table 2, $p = 0.0465$ and $p = 0.0318$). Succinate is a metabolite of the tricarboxylic acid cycle (TCA) cycle and an electron donor to complex II (Succinate-Q oxidoreductase) in oxidative metabolism. Glutamate is also the metabolic intermediate of glutaminolysis,

which would feed into the TCA cycle upstream of succinic acid at alpha-ketoglutarate. Interestingly, fructose-6-phosphate did not reach statistical significance (non-obese vs. obese ratio 1.62, $p = 0.0684$) but contributed to principle component analysis variance (VIP was 1.67). Fructose 6-phosphate is an important intermediate in glycolysis. Taken together, low AMP, succinate, glutamate, and fructose 6-phosphate suggest that KpB tumors in obese mice have a substantially altered metabolic phenotype compared to tumors that have arisen in non-obese controls. We are currently investigating the role of glycolysis and oxidative metabolism, along with AMPK and mTOR signaling, in ovarian cancers from obese and non-obese patients.

Finally, cytidine is a precursor of cytodinetriphosphate, which is needed to create phosphatidylcholine (PC) and phosphatidylethanolamine. Interestingly, lysoPC(16:1(9Z)) was also downregulated in the ovarian tumors from obese versus non-obese mice. Lysophospholipids (LPLs) can play a role in signaling through G-protein coupled receptors, and are a readily accessible fat source for cancer cells [42]. LPLs are generated via inflammatory-responsive phospholipase A (PLA) activity, suggesting that there may be altered inflammatory signaling between non-obese and obese tumors, which is currently being explored. In our genomic analysis, significant enrichment was found in "phospholipid binding" in the ovarian tumors from the obese mice, potentially corresponding to increased utilization of lysophospholipids in the setting of obesity and depletion of cytidine and lysoPC.

In conclusion, we demonstrate that the obese state can promote tumor progression in the KpB mouse model of serous OC, resulting in genomic and metabolic differences between tumors arising in the obese versus non-obese state. Our work suggests that the metabolic consequences of obesity may be crucial in the pathogenesis of OC, resulting in *biologically distinct* cancers than those that arise in normal weight women. This may have important implications for the treatment of this disease, such that obesity status may be a critical factor in the individualization of management strategies. Further work will be focused on the investigation of the identified obesity-dependent metabolic biomarkers as well as potential novel targets of treatment that may be specific to obesity-driven OCs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2013.12.026.

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